AD_____

Award Number: W81XWH-04-1-0371

TITLE: BRCA1 Protein Complexes: Dynamic Changes and Functions Important in Breast Cancer

PRINCIPAL INVESTIGATOR: Andrew Horwitz, Ph.D.

CONTRACTING ORGANIZATION: Harvard Medical School
Boston, MA 02115

REPORT DATE: April 2007

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
01-04-2007	Annual Summary	31 Mar 2004 – 30 Mar 2007

4. TITLE AND SUBTITLE

BRCA1 Protein Complexes: Dynamic Changes and Functions Important in Breast Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-04-1-0371

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Andrew Horwitz, Ph.D.

Email: ahorwitz@cmp.ucsf.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Harvard Medical School
Boston, MA 02115

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES
Original contains colored plates: ALL DTIC reproductions will be in black and white.

14. ABSTRACT

In this final report, I summarize the major accomplishments achieved during my three-year award period. Initial experiments focused on purification of endogenous BRCA1 complexes; however, the most fruitful work has occurred in characterizing the function of such BRCA1 complexes. I describe here distinct mechanisms for transcriptional stimulation and repression. These activities recapitulate the in vivo transcriptional functions of BRCA1.

15. SUBJECT TERMS
BRCA1, tumor suppressors, transcription

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	25	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

t

Intro

Over the course of the three-year award period, I have studied the function of BRCA1 complexes in a fully purified transcription system, and I have described distinct mechanisms for transcriptional stimulation and repression. These activities recapitulate the *in vivo* transcriptional functions of BRCA1, and indicate that transcriptional regulation by BRCA1 may be central to its role as a tumor suppressor.

Body

Task 1: To purify and characterize BRCA1 complexes from cultured mammalian cell lines.

A) Subcloning to assemble a retroviral zz-TEV fusion vector.
B) Infection and selection of stable cell lines
C) Purification by chromatography and affinity steps.
D) Identification of protein subunits by mass spectrometry and comparison between breast and non-breast cell line.

Several stable cell lines were previously established (as reported in year 1). Purification of endogenous complexes was attempted with mixed success. The interaction between BRCA1/BARD1 and the basal transcription machinery was further explored in Task 3.

Task 2: To describe the dynamics of BRCA1 complex formation and redistribution.

A) Purify BRCA1 complexes from cells synchronized at different points in the cell cycle or following DNA damage.
B) Compare distribution of complexes by Native Blue PAGE.

See Task 1.

Task 3: To test the function of purified BRCA1 complexes by *in vitro* assays.

Since the last report, I have continued characterization of the BRCA1/BARD1 interaction with RNA Polymerase II, and we have made great progress in this area. As reported previously, BRCA1/BARD1 ubiquitinate the large subunit of RNA Polymerase II (Rpb1), and we developed a fully-purified transcription/ubiquitination assay to ask whether the enzymatic activity of RNA Polymerase II was regulated by this modification. This assay demonstrates that ubiquitination of the pre-initiation-complex (PIC) by BRCA1/BARD1 induces dissociation of TFIIE, leading to a failure of initiation. Ion parallel to this work, we conducted gene expression profiling experiments, and demonstrated that acute shRNA knockdown of BRCA1 induces the expression of a number of gene targets, supporting a repressor role for BRCA1. As reported previously, BRCA1 also can stimulate transcription, independent of its E3 ubiquitin ligase activity. By stabilizing properly initiated PICs, BRCA1 promotes productive transcriptional initiation. In the cell, these opposing activities are likely regulated by interaction with the E2 enzyme, and targeted by transcription factors that participate in combinatorial regulation of BRCA1.

Key Research Accomplishments

• Established cell lines for affinity purification of BRCA1 complexes (completed).

• Characterized the *in vitro* ubiquitination of Pol II by BRCA1/BARD1; confirmed the results *in vivo* (completed).

• Developed an *in vitro* system to assay the transcriptional activity of BRCA1. Described distinct mechanisms for transcriptional repression and stimulation by BRCA1 (completed)

Reportable Outcomes

1) Stable cell lines expressing affinity-tagged BRCA1, Pol II components.

2) Starita LM.*, **Horwitz AA.***, Keogh MC., Ishioka C., Parvin JD., and Chiba N. BRCA1/BARD1 ubiquitinate phosphorylated RNA polymerase II. J Biol Chem 280(26): 24498-505 (2005). *equal contribution

3) **Horwitz, A.A**, Sankaran, S, Parvin JD. (2006) Direct stimulation of transcription initation by BRCA1 requires both its amino and carboxy termini. J Biol Chem 281(13) 8317-8320 (2006).

4) **Horwitz AA.,** Affar, EB., Heine, GF., Shi, Y., Parvin, JD. A mechanism for transcriptional repression dependent on the BRCA1 E3 ubiquitin ligase. Proc Natl Acad Sci USA. 1041(16): 6614-9 (2007).

Conclusions

The studies described here expand our understanding of how BRCA1 functions as a tumor suppressor. The primary area of investigation in the past year focused on BRCA1 complex function *in vitro*, in particular as it relates to the interaction between BRCA1/BARD1 and the Pol II complex. We previously confirmed that Pol II was a substrate for BRCA1/BARD1 *in vitro* and *in vivo* (.pdf attached). First, using a fully purified *in vitro* system, we showed that ubiquitination of RNA Polymerase II by BRCA1/BARD1 represses transcription initiation through dissociation of TFIIE/TFIIH (.pdf attached). Second, we have shown that BRCA1 also activates transcription, independent of its E3 ligase activity, and working at the initiation stage. The purified system reveals that BRCA1 regulates localization of the pre-initiation complex, stabilizing properly initiated complexes (.pdf attached). Thus, BRCA1 is a dual function transcriptional regulator that regulates the assembly the pre-initiation complex.

To examine BRCA1 activity in the cell, we used gene-profiling techniques to compare the transcriptomes of cells expressing wild type BRCA1 to those acutely depleted of BRCA1 (by shRNA). These studies demonstrated that BRCA1 suppresses transcription of a significant number of targets (in addition to its known activation targets), revealing a previously under-appreciated role in transcriptional repression.

Regulation of Pol II by BRCA1/BARD1 could effect tumor suppression in two ways. First, in the acute response to DNA damage, the repressive action of BRCA1/BARD1 may contribute to the global, transient repression of transcription. Second, by regulation of specific gene targets, BRCA1 may control a tumor-suppressive transcription program.

In closing, I would like to express my gratitude to the reviewers and administrators of the CDMRP for my graduate fellowship. I had an excellent graduate training experience, and owe this in part to the generous funding I received from the U.S. Army.

A mechanism for transcriptional repression dependent on the BRCA1 E3 ubiquitin ligase

Andrew A. Horwitz, El Bachir Affar, George F. Heine, Yang Shi, and Jeffrey D. Parvin

PNAS published online Apr 9, 2007;
doi:10.1073/pnas.0610481104

A mechanism for transcriptional repression dependent on the BRCA1 E3 ubiquitin ligase

Andrew A. Horwitz*, El Bachir Affar†, George F. Heine, Yang Shi, and Jeffrey D. Parvin‡

Department of Pathology, Harvard Medical School and Brigham and Women's Hospital, Boston, MA 02115

Edited by Inder M. Verma, The Salk Institute for Biological Studies, La Jolla, CA, and approved March 9, 2007 (received for review November 28, 2006)

Loss of function of the tumor suppressor protein BRCA1 is responsible for a high percentage of familial and also sporadic breast cancers. Early work identified a stimulatory transcriptional coactivator function for the BRCA1 protein, and more recently, BRCA1 has been implicated in transcriptional repression, although few examples of repressed genes have been characterized. We recently used an *in vitro* transcription assay to identify a biochemical mechanism that explained the BRCA1 stimulatory activity. In this study, we identified an ubiquitin-dependent mechanism by which BRCA1 inhibits transcription. BRCA1 ubiquitinates the transcriptional preinitiation complex, preventing stable association of TFIIE and TFIIH, and thus blocks the initiation of mRNA synthesis. What is striking about this mechanism of regulation by BRCA1 is that the ubiquitination of the preinitiation complex is not targeting proteins for degradation by the proteasome, nor are ubiquitin receptors modifying the activity, but rather the ubiquitin moiety itself interferes with the assembly of basal transcription factors at the promoter. Using RNAi to knockdown expression of the endogenous BRCA1 protein, we assessed the level of repression dependent on BRCA1 in the cell, and we found that BRCA1 is at least as significant a transcriptional repressor as it is an activator. These results define a biochemical mechanism by which the BRCA1 enzymatic activity regulates a key cellular process.

RNA polymerase II | TFIIE | transcription

BRCA1 is the breast and ovarian cancer specific tumor suppressor (1). Loss of BRCA1 can occur either by mutation of both alleles of the gene in the tumor cell (≈4% of all breast cancer cases) or by epigenetic down-regulation of the gene by methylation of its promoter (≈14% of sporadic breast cancer cases and up to 30% of ovarian cancer cases) (2, 3).

How BRCA1 protein exerts its tumor suppressor function remains unresolved, but it has been found to regulate a number of processes including transcription, repair of DNA damage, cell cycle checkpoints, and centrosome dynamics (4–6). The biochemical mechanism(s) by which BRCA1 regulates these diverse processes is unknown. The BRCA1 protein has the enzymatic activity of an E3 ubiquitin ligase when bound as a heterodimer to BARD1 (7, 8), and it is likely that the ubiquitin ligase activity is critical for BRCA1/BARD1 regulation of transcription and other processes. In this paper, the BRCA1/BARD1 heterodimer will be simply referred to as "BRCA1."

Previously, we found that BRCA1 strongly stimulated transcription by stabilizing the preinitiation complex (PIC) on the core promoter (9). This activity was observed in either the presence or absence of BARD1 and was independent of ubiquitination function. Our results suggested that BRCA1 enhanced the stability of the PIC on promoter elements relative to bulk DNA (9). Because BRCA1 can ubiquitinate phosphorylated RNA polymerase II (RNAPII) both *in vitro* and *in vivo* (10, 11), we wondered whether the E3 ubiquitin ligase activity of BRCA1 might alter its stimulatory effect on transcription. We find in these experiments that the E3 ubiquitin ligase activity of BRCA1 strongly inhibits transcription by blocking PIC assembly.

Results

Ubiquitin-Dependent Repression of Transcription. We tested the effects of BRCA1 E3 ubiquitin ligase activity in transcription reactions containing purified transcription and ubiquitination factors [TATA binding factor (TBP), TFIIB, RNAPII, TFIIF, TFIIE, TFIIH, E1, and E2 (UbcH5c)]. In the absence of the BRCA1/BARD1 heterodimer (BRCA1), the addition of ubiquitin had a negligible effect on RNA synthesis, and no ubiquitination of RNAPII was observed. However, when BRCA1 was included in the reaction, addition of ubiquitin repressed transcription nearly completely (Fig. 1*a Upper*, lanes 3 and 4). Transcriptional repression correlated with ubiquitination of the phosphorylated large subunit of RNAPII (Rpb1; Fig. 1*a Lower*, lanes 3 and 4). Importantly, BRCA1 exerts the repressive effect directly through its E3 activity. A mutant protein, BRCA1(I26A), which cannot bind the E2 enzyme (12), failed to ubiquitinate phospho-Rpb1 and did not repress transcription (lanes 5 and 6). These results link the enzymatic activity of BRCA1 to a previously unrecognized form of transcriptional repression.

The transcriptional repression depends on the inclusion in reactions of each ubiquitination factor. In otherwise complete reactions, we omitted one ubiquitination factor per reaction, and we found that repression of transcription required all of the factors [supporting information (SI) Fig. 6], and the level of repression correlated with BRCA1 concentration (SI Fig. 7).

Repression of Transcription Is Specific to BRCA1. The E3 ubiquitin ligase activity of BRCA1 exerted a strong repressive effect on RNA synthesis. Although TFIIH is another RING domain class of E3 ubiquitin ligase present in the transcription reaction (13), repression in this assay depends on the inclusion of BRCA1. To test further whether repression in this assay might be a general property of ubiquitin ligases, we assayed the E6AP E3 ubiquitin ligase for inhibition of transcription. E6AP is a HECT domain E3 that can use the same E2 enzyme (UbcH5c) *in vitro* as BRCA1 (14–16). Polyubiquitin chain formation assays confirmed that our preparation of E6AP was functional, and the activity observed in this nonspecific assay was similar to that of BRCA1 on a molar basis (data not shown). Unlike BRCA1, E6AP addition had no effect on transcription, even when added

Author contributions: A.A.H. and J.D.P. designed research; A.A.H. and G.F.H. performed research; E.B.A. and Y.S. contributed new reagents/analytic tools; A.A.H. analyzed data; and A.A.H. and J.D.P. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Abbreviations: RNAPII, RNA polymerase II; PIC, preinitiation complex; ML, major late.

*Present address: Department of Cellular and Molecular Pharmacology, University of California, 600 16th Street, San Francisco, CA 94158-2517.

†Present address: Faculty of Medicine, Montreal University Research Centre, Maisonneuve-Rosemont Hospital, 5415 Boulevard de l'Assomption, Montreal, QC, Canada H1T 2M4.

‡To whom correspondence should be addressed at: Department of Pathology, Brigham and Women's Hospital, New Research Building 630, 77 Avenue Louis Pasteur, Boston, MA 02115. E-mail: jparvin@rics.bwh.harvard.edu.

This article contains supporting information online at www.pnas.org/cgi/content/full/0610481104/DC1.

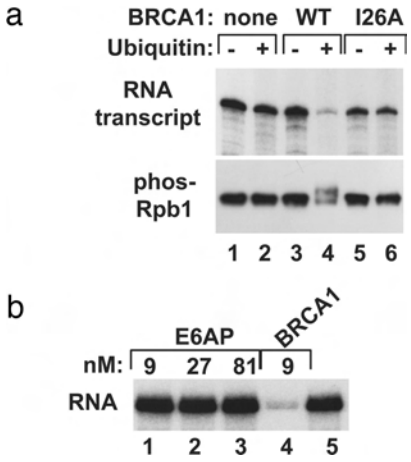

Fig. 1. The BRCA1 ubiquitin ligase ubiquitinates phosphorylated RNAPII and represses transcription. (*a*) Purified transcription/ubiquitination reactions containing TBP, TFIIB, RNAPII, TFIIF, TFIIE, and TFIIH and the ubiquitination factors E1 and E2 were assembled without BRCA1 (lanes 1 and 2), with full-length BRCA1 (lanes 3 and 4), or with the mutant BRCA1 protein (I26A) that is defective as an E3 ubiquitin ligase (lanes 5 and 6). Ubiquitin was included in reactions analyzed in lanes 2, 4, and 6. RNA products were analyzed (*Upper*). Immunoblots were probed with antibody against phosphorylated Rpb1 (*Lower*). (*b*) Transcription/ubiquitination reactions were assembled containing all factors except the E3 ubiquitin ligase, and RNA transcripts were analyzed. E6AP (lanes 1–3), BRCA1 (lane 4), or no E3 ubiquitin ligase (lane 5) were included in reactions at the indicated concentrations.

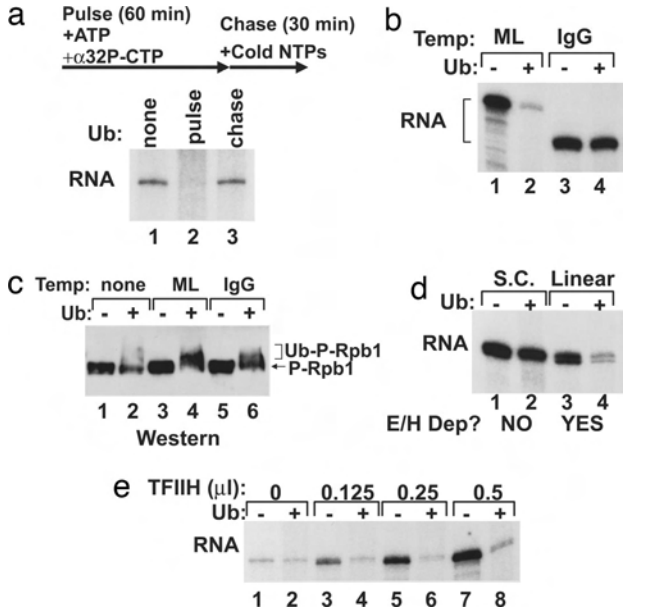

Fig. 2. BRCA1 E3 ubiquitin ligase represses initiation of transcription by blocking TFIIE and TFIIH function. (*a*) A pulse–chase protocol was used to separate the initiation and elongation phases. PICs in reactions containing the basal transcription factors, E1, E2, and BRCA1, were assembled during the pulse (UTP omitted) corresponding to the initiation phase. The addition of complete cold nucleotides in the chase allows elongation to proceed. Ubiquitin was omitted (lane 1) or added to otherwise complete transcription/ubiquitination reactions before the pulse (lane 2) or the chase (lane 3). RNA transcripts from each reaction are shown. (*b*) A comparison of the effect of the BRCA1 E3 ubiquitin ligase on transcription reactions from the adenoviral ML (lanes 1 and 2) and the IgG (lanes 3 and 4) promoters. Reactions were complete (basal transcription factors, E1, E2, and BRCA1) with the exception of ubiquitin, which was added to even-numbered lanes. RNA transcripts are shown. (*c*) In this immunoblot probed for phosphorylated Rpb1, the ubiquitination state of Rpb1 in the transcription/ubiquitination reaction was determined in the absence of template (lanes 1 and 2), with the ML template (lanes 3 and 4), and with the IgG template (lanes 5 and 6). Ubiquitin was added in even-numbered lanes. (*d*) BRCA1 E3 ubiquitin ligase repression of transcription was compared from negatively supercoiled (S.C.; lanes 1 and 2) and linear (lanes 3 and 4) IgG template. Ubiquitin was included in even-numbered lanes, and RNA transcripts are shown. Whether active transcription was dependent on the inclusion of TFIIE and TFIIH (E/H Dep?) is indicated for supercoiled and linear templates (17). (*e*) TFIIH was titrated into otherwise complete transcription/ubiquitination reactions: no TFIIH added (lanes 1 and 2), 0.125 μl (lanes 3 and 4), 0.25 μl (lanes 5 and 6), and 0.5 μl (lanes 7 and 8). Ubiquitin was included in even-numbered lanes, and resulting RNA transcripts are shown. Temp, template.

at 9-fold molar excess relative to BRCA1 (Fig. 1*b*). This result indicates that repression of transcription is specific to BRCA1 and not an effect of E3 ligases in general.

Repression of Transcription by BRCA1 Occurs During Initiation. We tested whether the repressive activity of BRCA1 affects a specific stage of transcription by use of a pulse–chase approach. Transcription/ubiquitination reactions containing all factors but ubiquitin were assembled without UTP. In the absence of UTP, transcription initiates but stalls within four nucleotides, producing nascent, radiolabeled transcripts. Transcriptional elongation continues when a cold chase mix of complete nucleotides is added to the reaction (Fig. 2*a*). New initiations that occur during the chase phase are unlabeled, and thus only the transcripts initiated during the pulse phase are detectable. When ubiquitin was omitted from the reaction, normal RNA synthesis occurred, demonstrating that the pulse–chase design does not inhibit transcription (Fig. 2*a*, lane 1). If ubiquitin was added before the pulse (initiation), complete repression of transcription resulted (Fig. 2*a*, lane 2). By contrast, if ubiquitin was added before the chase (elongation), no repression occurred (Fig. 2*a*, lane 3). Therefore, BRCA1 must act during the initiation phase to repress transcription. This is consistent with our prior observation that BRCA1 preferentially associates with and ubiquitinates the hyperphosphorylated form of Rpb1 associated with transcriptional initiation (10).

Promoter Specificity of Transcriptional Repression. We tested several promoters in the BRCA1 repression assay. Similar to transcription from the adenoviral major late (ML) promoter, the adenoviral E4 promoter was repressed by BRCA1 ubiquitination activity (data not shown). In stark contrast, transcription from the IgG promoter was immune to repression by BRCA1 and ubiquitination factors (Fig. 2*b*). The ubiquitination of phospho-RNAPII was stimulated by the presence of a template (Fig. 2*c*, compare lane 2 to lanes 4 and 6), suggesting that PIC formation was important for BRCA1-dependent ubiquitination. Surprisingly, even though ubiquitination of RNAPII did not inhibit transcription from the IgG promoter, the phospho-RNAPII was ubiquitinated to a similar extent as with the ML promoter (Fig. 2*c*).

Because Rpb1 was ubiquitinated to a similar extent in transcription reactions from both the ML and IgG template, the resistance of the IgG promoter to transcriptional repression by BRCA1 must involve another factor. Repression by the BRCA1 ubiquitin ligase occurs during the initiation phase of the transcription reaction, so we examined initiation factor requirements. Transcription from most promoters used in the *in vitro* assay is highly stimulated by TFIIE and TFIIH. This reflects the requirement for promoter melting during the initiation phase. The IgG promoter, however, is active in the absence of TFIIE and TFIIH when the template is negatively supercoiled. When the same template is linearized, the negative superhelical tension is released, and TFIIE and TFIIH are then required for active transcription initiation (17). Although transcription from the

supercoiled IgG template was resistant to repression, BRCA1 repressed transcription from a linear form of this plasmid (Fig. 2*d*). This finding indicates that ubiquitination of the transcription reaction by BRCA1 interferes with TFIIE and TFIIH function. We further tested this possibility by titrating TFIIH into transcription reactions with supercoiled ML plasmid template (Fig. 2*e*). Transcription from this template is weak in the absence of TFIIE and TFIIH but strongly stimulated by the addition of these basal factors. When TFIIH was omitted, transcription was minimal and the BRCA1 E3 ubiquitin ligase activity had no effect. Titration of TFIIH into the transcription reaction stimulated RNA synthesis in a dose-dependent manner in reactions lacking ubiquitin (Fig. 2*e*, odd lanes) but had no effect on reactions in which ubiquitin was included (even lanes). We conclude that the E3 ligase activity of BRCA1 blocks TFIIE and TFIIH function.

Although repression of transcription by BRCA1 is correlated with ubiquitination of phospho-Rpb1, the observation that TFIIE and TFIIH are the functional targets of the repression led us to investigate whether additional factors might be ubiquitinated. Western blot analysis of transcription reactions was limited by whether an available antibody could detect a given antigen at the concentration used in transcription reactions. Several basal factors were analyzed. We found that that the large subunit of TFIIE (p56E) is ubiquitinated by BRCA1 (Fig. 3*a*). In transcription reactions lacking template, more than one-half of the p56E was unmodified, and only monoubiquitination was apparent (Fig. 3*a*, lanes 1 and 2). The addition of either the ML or IgG template significantly enhanced ubiquitination levels, with multiubiquitination apparent (Fig. 3*a*, lanes 3–6). Thus, assembly of the transcription complex promotes ubiquitination of p56E, most likely by incorporating it into the PIC with BRCA1. Other factors, such as the RAP30 subunit of TFIIF (Fig. 3*a*, lower blot), were not targeted for ubiquitination, indicating that ubiquitination does not occur on all basal factors. Antibodies specific for TFIIH were insufficiently sensitive to determine whether any of the subunits of this factor were ubiquitinated in this reaction (data not shown).

Ubiquitination of RNAPII Blocks TFIIE Association with the PIC. During transcriptional initiation, TFIIE binds RNAPII and recruits TFIIH to the PIC (18). The finding that both Rpb1 and p56E are ubiquitinated in the transcription reaction led us to speculate that this modification might interfere with the stable association of TFIIE and RNAPII. Therefore we tested whether TFIIE binding to the transcription PIC was affected by BRCA1 E3 ligase activity. PICs were assembled on linear ML templates immobilized on agarose beads. In these reactions, ATP was the only nucleotide included and thus there could be no transcriptional elongation, but the RNAPII was phosphorylated, and ubiquitination was active. After incubation, the supernatant was collected, and the template was washed. The fractionation of p56E between the supernatant and template was monitored by Western blot analysis (Fig. 3*b*). The addition of ubiquitin to the PIC led to the appearance of ubiquitinated p56E in the supernatant (Fig. 3*b*, lane 2). In the bound fraction, the addition of ubiquitin reduced the amount of p56E associated with the template, and the remaining template-bound TFIIE was primarily unmodified (Fig. 3*b*, lane 4). Thus, ubiquitination of the PIC by BRCA1 interferes with TFIIE association, and this is likely the mechanism by which TFIIE/TFIIH function is blocked.

The dissociation of TFIIE from the transcription complex could occur because of ubiquitination of Rpb1, of p56E, or by some combination of the two. To distinguish the contribution of these modifications to transcriptional repression, we used a staged transcription assay with the immobilized template. In the first stage, PICs were assembled on linear template DNA with only ATP present. Ubiquitination factors were included in this

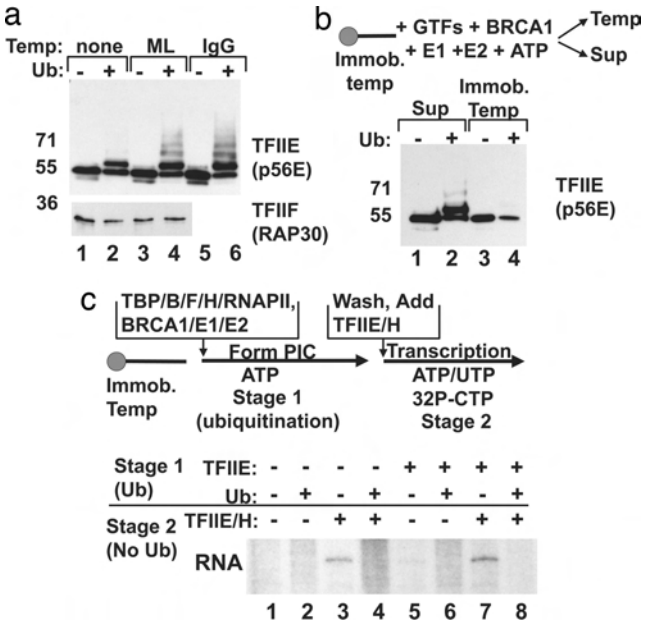

Fig. 3. BRCA1 ubiquitination of the PIC regulates the association of TFIIE. (*a Upper*) A Western blot of transcription reactions containing no template (lanes 1 and 2), ML template (lanes 3 and 4), and IgG template (lanes 5 and 6) was probed with TFIIE (p56E) antibody. (*a Lower*) A Western blot of transcription reactions containing no template (lanes 1 and 2) or ML template (lanes 3 and 4) was probed with TFIIF (RAP30)-specific antibody. (*b*) The fractionation of TFIIE (p56E) between the template (Temp) and supernatant (Sup) was analyzed by using an immobilized template (Immob. temp). PICs were assembled on bead-bound ML templates in the presence of the general transcription factors, BRCA1, E1, and E2 enzymes, and ATP. Reactions in lanes 2 and 4 also contained ubiquitin. The supernatant (lanes 1 and 2) was separated from the washed template (lanes 3 and 4), and the proteins were analyzed on protein gels. Western blots were probed with TFIIE (p56E) antibody. (*c*) A staged transcription protocol was used to test whether ubiquitination of a PIC lacking TFIIE could repress transcription. In Stage 1, transcription/ubiquitination reactions were assembled on an immobilized template in the presence of ATP only, permitting formation of the PIC and ubiquitination. The template was washed and incubated with complete nucleotides in Stage 2 plus TFIIE and TFIIH as indicated. Ubiquitin was included in even-numbered reactions in Stage 1. TFIIE was omitted from the Stage 1 reaction (lanes 1–4) or included (lanes 5–8). TFIIE and TFIIH were added back during Stage 2 (lanes 3, 4, 7, and 8), and transcription products were analyzed.

reaction mixture. In the second stage, the templates were washed to remove all unbound factors, including the soluble ubiquitination factors. The templates were then incubated in a new transcription mix containing the complete nucleotide mixture so that elongation could occur (Fig. 3*c*). The important feature of this experimental design is that ubiquitination is only possible during Stage 1 (supporting controls are shown in SI Fig. 8). When TFIIE was omitted from the reaction entirely, no RNA synthesis occurred (Fig. 3*c*, lanes 1 and 2). Addition of TFIIE in Stage 2 rescued transcription (Fig. 3*c*, lane 3) but not if ubiquitin was present in Stage 1 (Fig. 3*c*, lane 4). Therefore, addition of unmodified TFIIE to ubiquitinated PICs cannot rescue transcription. The results were the same if TFIIE was added in both stages of transcription (Fig. 3*c*, lanes 5–8). We propose that the E3 ligase activity of BRCA1 targets a region adjacent to the binding interface of TFIIE and Rpb1, and thus both proteins are ubiquitinated. However, from a functional standpoint, our experiments suggest that RNAPII is the key ubiquitination substrate blocking initiation. In addition, the ubiquitination of phospho-Rpb1 in *in vivo* and *in vitro* systems, both in the absence of and also stimulated by DNA damage, has been well documented (10, 19, 20). By contrast, we have not detected TFIIE

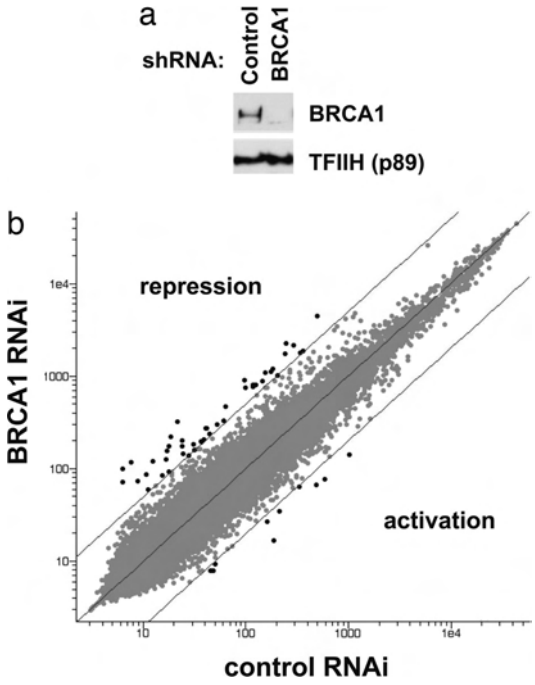

Fig. 4. RNAi knockdown of BRCA1 reveals a significant transcriptional repressor function. (*a*) HeLa cells were transfected with a plasmid expressing shRNA specific for GFP (control) or for BRCA1. Cells were harvested 72 h after transfection, and Western blots were probed with antibodies against BRCA1 (*Upper*) and the p89 subunit of TFIIH (loading control; *Lower*). (*b*) RNA isolated from the same cells (*a*) was used in microarray analysis. Genes with 5-fold or greater altered expression are highlighted in black. Those above the diagonal marking 5-fold effects were derepressed in the cells with knocked down BRCA1 expression.

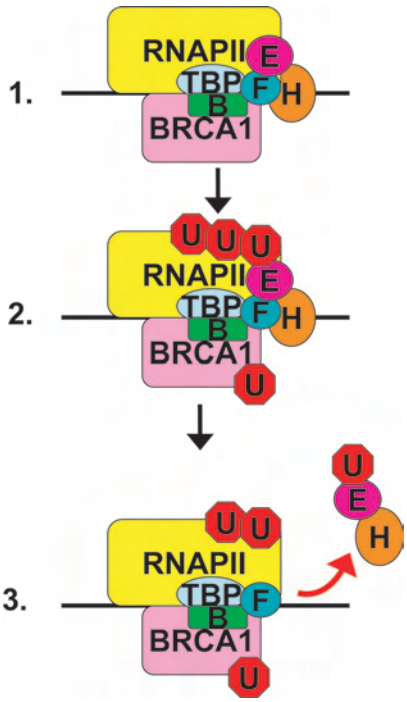

Fig. 5. Model diagram of BRCA1 repression of transcription: BRCA1 binds initiation competent PIC (1.), BRCA1 ubiquitinates itself, RNAPII, and TFIIE (2.), and TFIIE and TFIIH elute from the ubiquitinated PIC (3.).

ubiquitination in cells (data not shown), suggesting that phospho-RNAPII ubiquitination is the critical modification for the regulation of transcription by the BRCA1 E3 ubiquitin ligase.

Acute Silencing of BRCA1 Reveals a Large Number of Repressed Genes. The effects of BRCA1 on gene expression have largely been studied by overexpression of the BRCA1 protein in cells already expressing BRCA1 (for example, refs. 21 and 22). In these studies, exogenous expression of BRCA1 stimulated a large number of genes and repressed few genes. We found that after acutely silencing BRCA1 expression in HeLa cells using RNA interference, loss of BRCA1 resulted in higher expression of a large number of genes, indicating that BRCA1 repressed those targets (Fig. 4). Among the genes altered 2-fold or more, BRCA1 repressed ≈700 genes and stimulated ≈600 genes. Using a more stringent criterion of 5-fold effects, BRCA1 repressed 33 genes and stimulated eight. The effects of BRCA1 suppression on a number of these genes were confirmed by RT-PCR (SI Fig. 9). Although it is possible that many of the repressed genes were indirect targets of depletion of BRCA1, we suggest that the mechanism of ubiquitin-dependent repression of transcription identified in this study is an important component of the function of BRCA1 in the cell.

Discussion

In this study we found that BRCA1 represses transcription by preventing full assembly of the PIC (Fig. 5). BRCA1, in the absence of ubiquitin, binds to the PIC and even stimulates the level of transcription (9). Recruitment of the charged E2 to this promoter site would result in the ubiquitination of RNAPII, TFIIE, and also BRCA1. We have found that preubiquitinated BRCA1 protein does not disrupt the PIC (A.A.H., unpublished observations). Rather, the ubiquitination of the PIC, probably via phospho-Rpb1, results in the destabilization of TFIIE and TFIIH in the complex and the concomitant inactivation of transcription. The ubiquitinated RNAPII could still function in transcription on synthetic templates such as the IgG promoter under conditions in which TFIIE and TFIIH are not required. This is a previously unrecognized mode of transcriptional repression, whereby ubiquitination sterically blocks protein–protein association. Ubiquitination has been shown to affect a target protein by inducing binding to the proteasome (23, 24) or to other ubiquitin receptors (25). For the reaction described in this study, regulation by ubiquitin requires neither of these pathways but instead regulates the assembly of a multiprotein complex. Repression of transcription was observed here in the absence of proteasome or other ubiquitin receptors.

Does this mechanism, detected *in vitro*, operate in the cell? We argue that the answer is yes. Ubiquitinated phospho-RNAPII, although strongly stimulated by DNA damage, has also been detected in the undamaged cell (10, 19), and the overexpression of BRCA1 raises the level of RNAPII ubiquitination independent of DNA damage (10). These observations are consistent with a small percentage of promoters being repressed by BRCA1 ubiquitination of RNAPII. Whether the ubiquitinated RNAPII in the cell is subsequently degraded, because the proteasome would be present in such a setting, is unknown. BRCA1 has also been found to ubiquitinate RNAPII at 3′ processing sites of genes associated with the process of polyadenylation. In this latter case, the RNAPII is targeted for degradation (11). Clearly, BRCA1 interacts with the transcription apparatus, but it is uncertain whether the two identified mechanisms of BRCA1 regulation of transcription are mutually exclusive.

In combination with our previous study (9), these results demonstrate that BRCA1 regulates formation of the PIC, acting as a repressor or activator depending on the context. In the purified transcription assay, we controlled the switch between activation and repression by addition of the ubiquitination

factors. This may parallel the situation in the cell. Recent work indicates that the association of BRCA1 with its E2 enzyme is a regulated process (26), and we suggest that this could determine the activity of BRCA1 at a promoter. A second issue raised by our results is promoter specificity. In the *in vitro* system, protein concentrations are such that BRCA1 interacts with RNAPII directly. In the cell, however, we expect that protein partners of BRCA1 confer gene specificity. Sequence-specific factors, such as ZBRK1, c-Myc, and ERα, all recruit BRCA1 to genes for repression (27–33). In support of the concept that the promoter specificity of BRCA1 repression is due to specific DNA-binding factors, we located putative ZBRK1 binding sites in 19 of the 33 genes most repressed in our microarray study, but no identifiable ZBRK1 binding sites were observed in the genes stimulated by BRCA1 (data not shown). One function of BRCA1 in these repression complexes may be to recruit other repressors, such as CtIP (27), but the results shown herein using a defined transcription assay reveal that BRCA1 also has the capacity to directly regulate basal transcription factor function at the promoter.

How might the transcriptional repression of BRCA1 contribute to its function as a tissue-specific tumor suppressor? Several of the most significantly repressed targets identified in the initial microarray analysis are implicated in breast development or breast cancer (SI Fig. 9). Of particular interest are amphiregulin (AREG) and early growth response-1 (EGR-1). AREG is a ligand for the epidermal growth factor receptor (EGFR) that is essential for postnatal breast development (34) and overexpression of AREG has been noted in primary breast cancers (35). EGR-1 is a transcription factor with a variety of gene targets involved in angiogenesis, including EGFR (36, 37). Interestingly, high EGFR expression in breast cancers is correlated with loss of BRCA1 as well as poor clinical outcomes (38, 39). Based on the examples of repression of AREG and EGR-1 by BRCA1, we propose that transcriptional repression by BRCA1 may contribute to its tissue-specific tumor suppression.

Methods

Transcription Factors. The transcription factors used in these assays were purified by using established techniques (40–42).

Ubiquitination Factors. Full-length BRCA1/BARD1 were purified from baculovirus-infected insect cells as described (43). When indicated, the mutant BRCA1 containing isoleucine-26 to alanine substitution was purified by using the same methods as the wild type and was used in place of the wild-type protein. E1 and E2 (UbcH5c) were expressed in bacteria and purified (43). Bovine ubiquitin was purchased from Sigma (St. Louis, MO).

Plasmid Templates. G-less cassette templates were based on the p(C2AT)$_{19}$ vector (44) and have been described (45). The linearized IgG template was prepared by digestion with XmnI and subsequent gel purification. The immobilized ML template was prepared by excising the template from its plasmid with HindIII/XmnI digest. The fragment was gel purified, and the 5′ overhang produced by HindIII digestion was filled in with Biotin-14-dATP (Invitrogen, Carlsbad, CA) using the Klenow fragment of DNA Polymerase I. The excised DNA template was immobilized on streptavidin M-280 beads according to the manufacturer's directions (Dynal, Great Neck, NY).

Transcription/Ubiquitination Assay. Transcription assays were based on reactions described (9). Reactions contained 20 mM Hepes-NaOH (pH 7.9), 20% glycerol, 1 mM EDTA, 60 mM KCl, 0.1 mM each ATP and UTP, 0.05 mM 3′-*O*-methyl-GTP, 0.003 mM CTP, 1 mM DTT, 0.15 mg/ml BSA, 2 mM $MgCl_2$, 0.003 mM $ZnSO_4$, 1.2 mg/ml plasmid template (1 nM), 10 μCi (1 Ci = 37 GBq) of [α-^{32}P]CTP (800 Ci/mmol; PerkinElmer, Boston, MA), and transcription factors. Unless otherwise noted, the amounts of each factor used per 25-μl reaction were as follows: 8 ng of yeast TATA box-binding protein (16 nM), 60 ng of TFIIB (60 nM), 100 ng of calf thymus RNAPII, 100 ng of TFIIF (40 nM), 4 ng of TFIIE (1.8 nM), and 0.5 μl of TFIIH fraction. Ubiquitination factors were included in the reaction mixture or added to individual reactions before incubation: BRCA1 (9 nM; 50 ng of BRCA1 and 24 ng of BARD1), 6× His-E1 ubiquitin ligase (40 nM), 0.75 μg of 6× His-UbcH5c (2 μM), and 2 μg of ubiquitin (12 μM).

Reactions were assembled on ice and then incubated at 30°C for 90–120 min. Reactions were terminated by addition of 200 μl of transcription stop mix (7 M urea, 0.5% SDS, 2 mM EDTA, 0.1 M LiCl, 0.35 M NH_4OAc), extracted in phenol/chloroform, precipitated in ethanol, and resolved on 6% polyacrylamide gels containing 8.3 M urea. Gels were dried and exposed to film with an intensifying screen. PhosphorImager analysis was performed by using a Molecular Dynamics (Sunnyvale, CA) PhosphorImager and ImageQuant software.

Preparation of cDNA for Microarray and TaqMan Analysis. HeLa cells were cotransfected (Lipofectamine; Invitrogen) with 5 μg of shRNA expression plasmid (46) and 20 ng of pBabe-puro. BRCA1 shRNA (gccacaggaccccaagaatgag) was targeted to the 3′ untranslated region. The control shRNA was targeted against a mutant GFP construct (gggccatggcacgtacggcaag). Puromycin selection (3 μg/ml) was applied 24 h after transfection, and cells were harvested after 72 h. RNA was prepared with Tri Reagent (Molecular Research, Cincinnati, OH) and further purified over RNeasy columns (Qiagen, Valencia, CA). Microarray analysis was performed on separate samples at the Harvard Biopolymers Facility on the Affymetrix (Santa Clara, CA) HG-U133_plus_2 chip. For TaqMan assays, 10 ng of cDNA template was used in standard reactions.

We thank Karen Daniels and Matthew Spaits for technical assistance and R. Baer (Columbia University, New York, NY) for E6AP expression plasmid. This work was supported by a predoctoral fellowship from the Department of Defense Breast Cancer Research Program (to A.A.H.) and National Institutes of Health Grant CA90281 (to J.D.P.).

1. Miki Y, Swensen J, Shattuck-Eidens D, Futreal PA, Harshman K, Tavtigian S, Liu Q, Cochran C, Bennett LM, Ding W, *et al.* (1994) *Science* 266:66–71.
2. Rahman N, Stratton MR (1998) *Annu Rev Genet* 32:95–121.
3. Turner N, Tutt A, Ashworth A (2004) *Nat Rev Cancer* 4:814–819.
4. Starita LM, Parvin JD (2003) *Curr Opin Cell Biol* 15:345–350.
5. Venkitaraman AR (2002) *Cell* 108:171–182.
6. Parvin JD, Sankaran S (2006) *Cell Cycle* 5:1946–1950.
7. Wu LC, Wang ZW, Tsan JT, Spillman MA, Phung A, Xu XL, Yang MC, Hwang LY, Bowcock AM, Baer R (1996) *Nat Genet* 14:430–440.
8. Hashizume R, Fukuda M, Maeda I, Nishikawa H, Oyake D, Yabuki Y, Ogata H, Ohta T (2001) *J Biol Chem* 276:14537–14540.
9. Horwitz AA, Sankaran S, Parvin JD (2006) *J Biol Chem* 281:8317–8320.
10. Starita LM, Horwitz AA, Keogh MC, Ishioka C, Parvin JD, Chiba N (2005) *J Biol Chem* 280:24498–24505.
11. Kleiman FE, Wu-Baer F, Fonseca D, Kaneko S, Baer R, Manley JL (2005) *Genes Dev* 19:1227–1237.
12. Brzovic PS, Keeffe JR, Nishikawa H, Miyamoto K, Fox D, III, Fukuda M, Ohta T, Klevit R (2003) *Proc Natl Acad Sci USA* 100:5646–5651.
13. Takagi Y, Masuda CA, Chang WH, Komori H, Wang D, Hunter T, Joazeiro CA, Kornberg RD (2005) *Mol Cell* 18:237–243.
14. Wu-Baer F, Lagrazon K, Yuan W, Baer R (2003) *J Biol Chem* 278:34743–34746.
15. Scheffner M, Huibregtse JM, Howley PM (1994) *Proc Natl Acad Sci USA* 91:8797–8801.
16. Huang L, Kinnucan E, Wang G, Beaudenon S, Howley PM, Huibregtse JM, Pavletich NP (1999) *Science* 286:1321–1326.
17. Parvin JD, Sharp PA (1993) *Cell* 73:533–540.
18. Maxon ME, Goodrich JA, Tjian R (1994) *Genes Dev* 8:515–524.

19. Ratner JN, Balasubramanian B, Corden J, Warren SL, Bregman DB (1998) *J Biol Chem* 273:5184–5189.
20. Lee KB, Wang D, Lippard SJ, Sharp PA (2002) *Proc Natl Acad Sci USA* 99:4239–4244.
21. Harkin DP, Bean JM, Miklos D, Song YH, Truong VB, Englert C, Christians FC, Ellisen LW, Maheswaran S, Oliner JD, Haber DA (1999) *Cell* 97:575–586.
22. MacLachlan TK, Somasundaram K, Sgagias M, Shifman Y, Muschel RJ, Cowan KH, El-Deiry WS (2000) *J Biol Chem* 275:2777–2785.
23. Coux O, Tanaka K, Goldberg AL (1996) *Annu Rev Biochem* 65:801–847.
24. Voges D, Zwickl P, Baumeister W (1999) *Annu Rev Biochem* 68:1015–1068.
25. Katzmann DJ, Odorizzi G, Emr SD (2002) *Nat Rev Mol Cell Biol* 3:893–905.
26. Polanowska J, Martin JS, Garcia-Muse T, Petalcorin MI, Boulton SJ (2006) *EMBO J* 25:2178–2188.
27. Furuta S, Wang JM, Wei S, Jeng YM, Jiang X, Gu B, Chen PL, Lee EY, Lee WH (2006) *Cancer Cell* 10:13–24.
28. Zheng L, Pan H, Li S, Flesken-Nikitin A, Chen PL, Boyer TG, Lee WH (2000) *Mol Cell* 6:757–768.
29. Fan S, Wang J, Yuan R, Ma Y, Meng Q, Erdos MR, Pestell RG, Yuan F, Auborn KJ, Goldberg ID, Rosen EM (1999) *Science* 284:1354–1356.
30. Zheng L, Annab LA, Afshari CA, Lee WH, Boyer TG (2001) *Proc Natl Acad Sci USA* 98:9587–9592.
31. Wang Q, Zhang H, Kajino K, Greene MI (1998) *Oncogene* 17:1939–1948.
32. Xiong J, Fan S, Meng Q, Schramm L, Wang C, Bouzahza B, Zhou J, Zafonte B, Goldberg ID, Haddad BR, *et al.* (2003) *Mol Cell Biol* 23:8668–8690.
33. Kennedy RD, Gorski JJ, Quinn JE, Stewart GE, James CR, Moore S, Mulligan K, Emberley ED, Lioe TF, Morrison PJ, *et al.* (2005) *Cancer Res* 65:10265–10272.
34. Sternlicht MD, Sunnarborg SW, Kouros-Mehr H, Yu Y, Lee DC, Werb Z (2005) *Development (Cambridge, UK)* 132:3923–3933.
35. Desruisseau S, Palmari J, Giusti C, Romain S, Martin PM, Berthois Y (2004) *Int J Cancer* 111:733–740.
36. Fahmy RG, Dass CR, Sun LQ, Chesterman CN, Khachigian LM (2003) *Nat Med* 9:1026–1032.
37. Nishi H, Nishi KH, Johnson AC (2002) *Cancer Res* 62:827–834.
38. Lakhani SR, Reis-Filho JS, Fulford L, Penault-Llorca F, van der Vijver M, Parry S, Bishop T, Benitez J, Rivas C, Bignon YJ, *et al.* (2005) *Clin Cancer Res* 11:5175–5180.
39. Sainsbury JR, Farndon JR, Sherbet GV, Harris AL (1985) *Lancet* 1:364–366.
40. Haile DT, Parvin JD (1999) *J Biol Chem* 274:2113–2117.
41. Mondal N, Parvin JD (2001) *Nature* 413:435–438.
42. Schlegel BP, Green VJ, Ladias JA, Parvin JD (2000) *Proc Natl Acad Sci USA* 97:3148–3153.
43. Starita LM, Machida Y, Sankaran S, Elias JE, Griffin K, Schlegel BP, Gygi SP, Parvin JD (2004) *Mol Cell Biol* 24:8457–8466.
44. Sawadogo M, Roeder RG (1985) *Cell* 43:165–175.
45. Parvin JD, Shykind BM, Meyers RE, Kim J, Sharp PA (1994) *J Biol Chem* 269:18414–18421.
46. Sui G, Soohoo C, Affar el B, Gay F, Shi Y, Forrester WC (2002) *Proc Natl Acad Sci USA* 99:5515–5520.

ACCELERATED PUBLICATION

This paper is available online at www.jbc.org

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 281, NO. 13, pp. 8317–8320, March 31, 2006

Direct Stimulation of Transcription Initiation by BRCA1 Requires Both Its Amino and Carboxyl Termini*

Received for publication, December 21, 2005, and in revised form, January 27, 2006
Published, JBC Papers in Press, February 10, 2006, DOI 10.1074/jbc.C500475200

Andrew A. Horwitz[‡], Satish Sankaran[§], and Jeffrey D. Parvin[§1]

From the [‡]Program in Biology and Biomedical Sciences, Harvard Medical School, Boston, Massachusetts 02115 and the [§]Department of Pathology, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts 02115

Published experiments suggest that BRCA1 interaction with RNAPII and regulation of a number of target genes may be central to its role as a tumor suppressor. Previous *in vivo* and *in vitro* work has implicated the carboxyl terminus of BRCA1 in transcriptional stimulation, but the mechanism of action remains unknown, and whether the full-length protein stimulates transcription is controversial. BRCA1 interacts with a number of enhancer-binding transcriptional activators, suggesting that these factors recruit BRCA1 to promoters, where it stimulates RNA synthesis. To investigate whether BRCA1 has intrinsic transcriptional activity, we established a fully purified transcription assay. We demonstrate here that BRCA1 stimulates transcription initiation across a range of promoters. Both the amino and carboxyl termini of BRCA1 are required for this activity, but the BRCA1-binding partner, BARD1, is not. Our data support a model whereby BRCA1 stabilizes productive preinitiation complexes and thus stimulates transcription.

Of the many functions attributed to BRCA1,[2] one of the first identified was transcriptional stimulation (1, 2). BRCA1 copurifies with the RNA polymerase II (RNAPII) holoenzyme (3, 4), and reporter assays and microarray studies show that it regulates the expression of a range of p53-dependent and -independent targets (5, 6). Thus, one way in which BRCA1 may serve as a tumor suppressor is through up-regulation of growth-suppressive targets (7, 8). While the mechanism of stimulation is unknown, the transcriptional activity of BRCA1 most likely depends in part on its reported interactions with a wide range of transcriptional activators. However, in a defined system assayed *in vitro*, a Gal4 fusion to the carboxyl terminus of BRCA1 activates transcription, independent of other activators (9), suggesting an intrinsic transcriptional activity for BRCA1. A subsequent study found that Gal4 fusions to full-length BRCA1 could not activate transcription in transfected cells and that the degree of transcriptional activation conferred by Gal4 fusions to the carboxyl terminus of bovine BRCA1 was much lower than human BRCA1 (10). Since the human carboxyl terminus is more acidic than the bovine version, the transcriptional activity may simply be a function of its acidity. Regardless, *in vivo* reporter assays using BRCA1 without a Gal4 fusion indicate that transcriptional stimulation by BRCA1 is dependent on its carboxyl terminus (6, 11). To better understand whether BRCA1 might directly regulate transcription, we developed an assay to test the function of full-length human BRCA1 in transcription, independent of an artificial DNA-binding domain protein fusion. We demonstrate here that BRCA1 stimulates basal transcription by promoting initiation of RNA synthesis. This is the first demonstration of direct transcriptional activity by full-length BRCA1.

* This work was supported by a predoctoral fellowship from the Department of Defense Breast Cancer Research Program (to A. A. H.), a postdoctoral fellowship from the Komen Foundation (to S. S.), and National Institutes of Health Research Grant CA90281 (to J. D. P.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] To whom correspondence should be addressed. E-mail: jparvin@rics.bwh.harvard.edu.

[2] The abbreviations used are: BRCA1, breast cancer gene 1; BARD1, BRCA1-associated RING domain protein 1; RNAPII, RNA polymerase II; TBP, TATA-binding protein; TFII, transcription factor II.

MATERIALS AND METHODS

Transcription Factors—The transcription factors used in these assays were purified using established techniques (9, 12, 13). BRCA1/BARD1, BRCA1, and the truncation mutants were purified from baculovirus infected insect cells as described previously (14, 15). p53 was also purified from baculovirus infected insect cells (16).

Plasmid Templates—G-less cassette templates were based upon the $p(C2AT)_{19}$ vector (17) and have been described previously (18).

Transcription Assay—Transcription assays were based on reactions described by Parvin and Sharp (19). Reactions contained 20 mM Hepes-NaOH, pH 7.9, 20% glycerol, 1 mM EDTA, 60 mM KCl, 0.1 mM each ATP and UTP, 0.05 mM 3′-*O*-methyl-GTP, 0.003 mM CTP, 1 mM dithiothreitol, 0.15 mg/ml bovine serum albumin, 2 mM $MgCl_2$, 0.003 mM $ZnSO_4$, 1.2 μg/ml plasmid template (1 nM), 10 μCi of [α-^{32}P]CTP (800 Ci/mmol; PerkinElmer Life Sciences) and transcription factors. Unless otherwise noted, the amount of each factor used per 25-μl reaction was: 8 ng of yeast TBP (16 nM) or 1 μl of immunoaffinity-purified TFIID (containing ~4 ng of TBP), 60 ng of TFIIB (60 nM), 100 ng of TFIIA (60 nM), 100 ng of calf thymus RNA polymerase II, 100 ng of TFIIF (40 nM), 4 ng of TFIIE (1.8 nM), and 0.5 μl of TFIIH fraction. Transcriptional activation reactions with p53 contained 100 ng of PC4 (270 nM). Reactions were assembled on ice and then incubated at 30 °C for 120 min. Reactions were terminated by addition of 200 μl of transcription stop mix (7 M urea, 0.5% SDS, 2 mM EDTA, 0.1 M LiCl, 0.35 M NH_4OAc), phenol/chloroform-extracted, ethanol-precipitated, and resolved on 6% polyacrylamide gels containing 8.3 M urea. Gels were dried and exposed to film with an intensifying screen. PhosphorImager analysis was performed using an Amersham Biosciences PhosphorImager and ImageQuant software.

RESULTS AND DISCUSSION

Based on the prior evidence that BRCA1 is a coactivator of p53 transcriptional targets (5, 6), we first attempted to reconstitute coactivation by purified full-length BRCA1/BARD1 and p53 *in vitro*. We reasoned that in the absence of a Gal4 fusion, sequence specific p53 binding might serve to localize BRCA1/BARD1 to the promoter region. Transcription reactions were performed with purified TFIID, TFIIB, TFIIA, RNAPII, TFIIE, TFIIF, TFIIH, and PC4. To detect transcriptional activation, a modified adenoviral E4 promoter with upstream p53 response elements (p53 G5E4) linked to a 384-base pair G-less cassette was used (16). As an internal control template for basal transcription, the adenoviral major late promoter (ΔML) linked to a 210-base pair G-less cassette was used. Transcription from both templates was low in the absence of BRCA1/BARD1 and p53 (Fig. 1*A*, *lane 1*). To our surprise, addition of BRCA1/BARD1 alone stimulated transcription from both templates (*lane 2*). Addition of p53 specifically activated transcription of the p53 G5E4 template (*lane 3*). Addition of both p53 and BRCA1/BARD1 resulted in the highest ratio of activated/basal transcription, demonstrating that a modest amount of coactivation can occur with these purified factors (*lane 4*). We were intrigued that BRCA1/BARD1 could stimulate transcription in the absence of p53 or a Gal4 fusion. In the following experiments we characterized the mechanism by which BRCA1 directly stimulates basal transcription.

In addition to leaving out p53, we found that by omitting PC4, a factor required for activated transcription (20), the level of RNA synthesis was significantly higher and the stimulatory effect on transcription by BRCA1 was apparent (Fig. 1*B*). We tested several promoters for effects by BRCA1/BARD1 on RNA synthesis. All of these templates were identical with the exception of the 50 base pairs of sequence in the core promoter immediately upstream of the G-less cassette sequence. The magnitude of the stimulation of RNA synthesis by BRCA1/BARD1 differed among templates, indicating that the effect of BRCA1/BARD1 varied dependent on core promoter sequences (Fig. 1*B*). Stimulation was highest (~10-fold) for the p53 G5E4 promoter template (*lanes 5* and *6*), and we chose that template for subsequent experiments. The fact that BRCA1/BARD1 stimulated transcription from the IgG template, which does not require TFIIE/TFIIH, indicated that these factors were not required for transcriptional stimulation. Indeed, removal of TFIIE and TFIIH from the reaction and substitution of TBP for TFIID did not affect the stimulation of RNA synthesis by BRCA1/BARD1 (Fig. 1*C*).

One trivial explanation for these results would be if the BRCA1/BARD1 preparation used in our assay contained a contaminating general transcription

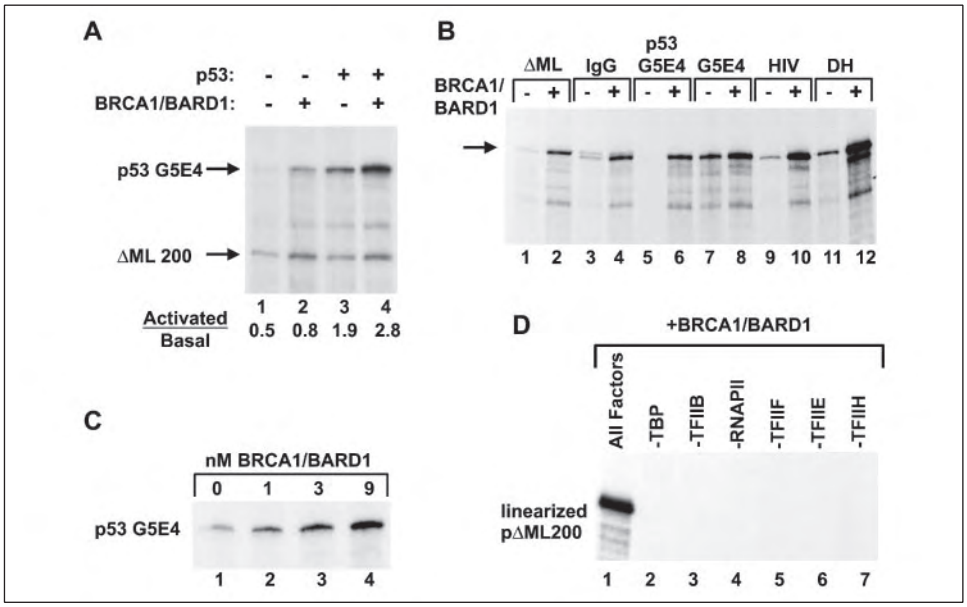

FIGURE 1. **BRCA1/BARD1 stimulates basal transcription.** *A*, BRCA1/BARD1 (10 nM) or p53 (40 nM) were added to *in vitro* transcription reactions containing purified factors (TFIID, TFIIA, TFIIB, RNAPII, TFIIF, TFIIE, and TFIIH) and the coactivator PC4. Transcription from the p53 G5E4 plasmid, which contains a p53 response element, yields a 390-nucleotide RNA. Transcription from the basal control template, ΔML200, which lacks p53 response elements, yields a 210-nucleotide RNA. Basal transcription in the absence of activators (*lane 1*) or stimulation of transcription by p53 (*lanes 3* and *4*) and BRCA1/BARD1 (*lanes 2* and *4*) were assayed. The ratio of stimulated/basal transcription was determined by PhosphorImager analysis of the accumulated RNA from the p53 G5E4 template relative to the ΔML200 template. *B*, stimulation of transcription by BRCA1/BARD1 was tested in reactions containing TFIID, TFIIA, TFIIB, RNAPII, TFIIF, TFIIE, and TFIIH using a variety of single ~400-base pair G-less cassette templates. Full-length transcript is noted by an *arrow*. The following promoters linked to G-less cassette templates were used: ΔML, the core adenoviral major late promoter (*lanes 1* and *2*); IgG, the immunoglobulin heavy chain promoter (*lanes 3* and *4*); p53 G5E4, the adenoviral E4 promoter with a 20-bp p53 response element upstream of the TATA box (*lanes 5* and *6*); G5E4, the adenoviral E4 promoter without p53 response elements (*lanes 7* and *8*); HIV, the human immunodeficiency virus promoter (*lanes 9* and *10*); DH, the *Drosophila* heat shock promoter (*lanes 11* and *12*). *C*, transcriptional stimulation by BRCA1/BARD1 in a minimal system, including TBP, TFIIA, TFIIB, RNAPII, TFIIF and the p53 G5E4 template. BRCA1/BARD1 was omitted (*lane 1*) or added at 1–9 nM concentrations, as indicated (*lanes 2–4*). *D*, the BRCA1/BARD1 preparation does not complement transcription reactions lacking a single factor. Transcriptions from a linearized ΔML200 template were assembled with BRCA1/BARD1 and TBP, TFIIB, RNAPII, TFIIF, TFIIE, and TFIIH (*lane 1*). *Lanes 2–7* were assembled in the same way but with a single transcription factor omitted: TBP (*lane 2*), TFIIB (*lane 3*), RNAPII (*lane 4*), TFIIF (*lane 5*), TFIIE (*lane 6*), and TFIIH (*lane 7*).

factor that was limiting in the assay. The BRCA1/BARD1 protein was purified from insect cells and judged free of major contaminants by silver stained protein gels (15). However, to rule out this possibility, we tested whether the BRCA1/BARD1 preparation could complement transcription reactions lacking a single factor (Fig. 1*D*). Transcriptions were conducted using a linearized ΔML template that requires TBP, TFIIB, RNAPII, TFIIF, TFIIE, and TFIIH. BRCA1/BARD1 was present in all reactions at a 9 nM concentration. Transcription was observed only when all factors were present, and thus we exclude the possibility that the BRCA1/BARD1 preparation contained a general transcription factor.

Having established that BRCA1/BARD1 stimulated basal transcription in a minimal RNAPII transcription system, we next asked what stage of transcription BRCA1/BARD1 enhanced. We used a pulse/chase strategy to separate transcriptional initiation from elongation (Fig. 2*A*). In the pulse phase, only ATP and [α-^{32}P]CTP were added to the reaction mixture. The lack of UTP prevented elongation from occurring beyond four nucleotides, resulting in a stalled RNAPII complex. In the chase phase, a complete, unlabeled nucleotide mixture was added with excess CTP, allowing elongation of the labeled nascent transcripts. Any new initiations that occurred during the chase phase were unlabeled and thus not detected. Regardless of whether TFIID or TBP was used for TATA binding activity, inclusion of BRCA1/BARD1 during the pulse stimulated transcription, while addition during the chase had no effect (Fig. 2*B*). These results indicated that BRCA1/BARD1 stimulate basal transcription by promoting initiation. However, it was also possible that BRCA1/BARD1 load during the initiation phase but then promote transcriptional elongation. To determine whether this might be true, we examined transcription from very short templates (40–50 nucleotides), reasoning that the importance of an elongation factor over such a short template would be greatly reduced. A similar level of stimulation of RNA synthesis was observed for these mini-templates (~10-fold) as was seen for the ~400-base pair templates, thus supporting the idea that BRCA1/BARD1 promote the initiation of transcription (Fig. 2*C*).

Both BRCA1 and BARD1 copurify with the RNAPII holoenzyme (21), and thus we used the heterodimer in experiments to this point. The major functional outcome of the BRCA1/BARD1 interaction is to potentiate the E3 ubiquitin ligase activity of BRCA1 (22). We had no reason to believe this enzymatic function had a role in transcriptional stimulation because E1 and E2 enzymes and ubiquitin were omitted from the reactions. Therefore, we tested whether BARD1 was required for transcriptional stimulation by BRCA1. When comparing BRCA1/BARD1 to BRCA1 alone, we observed similar levels of stimulation of RNA synthesis, evident in each case at concentrations as low as 1 nM (Fig. 3*A*). We conclude that BARD1 is not required for transcriptional stimulation by BRCA1. Next we examined truncations of BRCA1 to determine what portion of the protein contains the stimulatory activity. Deletion of either the 300 amino-terminal residues or the 336 carboxyl-terminal residues of BRCA1 abolished stimulation of transcription (Fig. 3*B*). Both the amino and carboxyl termini of BRCA1 are known to interact with RNAPII (21), and these truncations may reduce association with RNAPII in our assay. In addition, previous reports localize transcriptional activity to the carboxyl terminus of BRCA1 (2, 9). Since truncation of either terminus did not support transcriptional stimulation, we tested an additional two internal deletions spanning most of the intervening sequence (Fig. 3*C*). Both BRCA1-(Δ303–770)/BARD1 and BRCA1-(Δ770–1290)/BARD1 stimulated transcription as well as or better than BRCA1/BARD1. At the highest concentration tested (9 nM), the BRCA1-(Δ770–1290)/BARD1 actually repressed transcription, possibly reflecting a transcriptional squelching effect. In summary, the amino and carboxyl termini, but not internal domains of BRCA1, are required for transcriptional stimulation (Fig. 3*D*).

Our data to this point suggested that BRCA1 might be promoting formation of the initiation complex through contacts mediated by its amino and carboxyl termini. To determine which transcription factors might be affected by these contacts, we attempted to titrate factors downward in concentration, reasoning that the stimulatory activity should be enhanced by limiting conditions for the relevant factors. To our surprise, downward titration of TFIIA resulted in higher levels of basal transcription and a reduction in the stimulatory effect of BRCA1 (Fig. 4*A*). Without TFIIA (*lanes 1* and *2*), we observed a negligible stimulatory effect of BRCA1, but the inhibitory activity of TFIIA on basal transcription was relieved by addition of BRCA1. TFIIA is known to act as an anti-repressor for TBP-binding inhibitors and is a required factor in activated transcription systems utilizing TFIID (23, 24), so repression was unexpected.

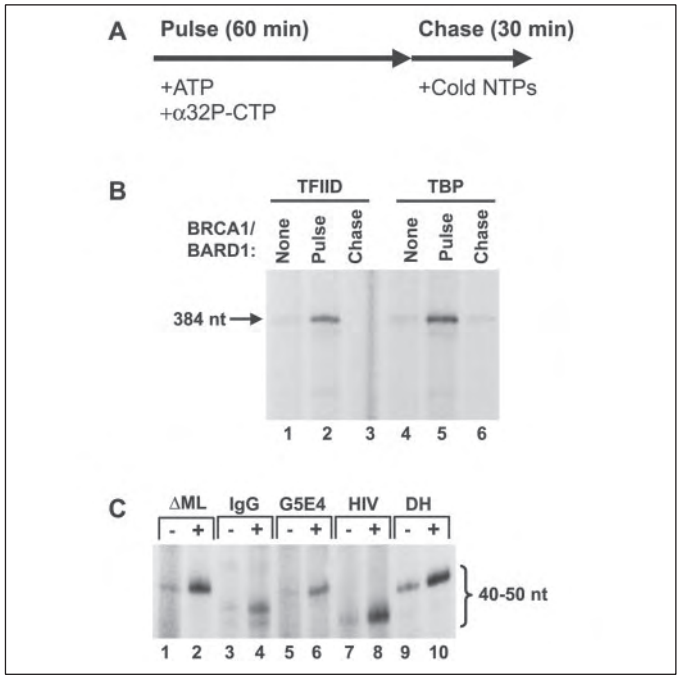

FIGURE 2. **BRCA1/BARD1 stimulate transcriptional initiation.** *A*, schematic of pulse/chase experiment used to separate transcriptional initiation from elongation. Transcription reactions were assembled without UTP and incubated for 60 min (pulse). Complete cold nucleotides, with excess CTP, were added for an additional 30-min incubation (chase). *B*, transcription reactions containing TFIIA, TFIIB, RNAPII, TFIIF, TFIIE, TFIIH, and TFIID (*lanes 1–3*) or TBP (*lanes 4–6*) were assembled as described above. BRCA1/BARD1 (10 nM) was omitted (*lanes 1* and *4*), added during the pulse (*lanes 2* and *5*), or added during the chase (*lanes 3* and *6*). *C*, transcription reactions containing TFIID, TFIIA, TFIIB, RNAPII, TFIIF, TFIIE, and TFIIH and mini-templates (~50 base pairs) were performed with (*even lanes*) or without (*odd lanes*) BRCA1/BARD1 (10 nM). The promoters correspond to those used with the ~400-base pair templates described in the legend to Fig. 1*B*.

However, this was not the first observation of basal repressive action by TFIIA. Prior to the cloning and recombinant expression of TFIIA, researchers reported on a repressive activity that purified closely with TFIIA (25). This activity repressed basal transcription from consensus TATA box promoters but stimulated transcription from non-consensus promoters. The authors (25) suggested a model whereby TFIIA interacts with TBP, altering its conformation and association with the promoter. If this conformational change altered the preference of TBP for the TATA box, then it could interfere with formation of the preinitiation complex on the correct DNA site and repress transcription (25).

Based on these previous findings, we speculated that BRCA1 might prevent improper TBP localization, either by disrupting non-TATA bound TBP or by stabilizing complex formation on *bona fide* TATA boxes. Precedent for regulation of TBP binding exists in the ATPase Mot1, which can dissociate TBP from DNA. Initial *in vitro* work cast Mot1 as a transcriptional inhibitor (26, 27), but examination *in vivo* also demonstrated activation of several targets (28–30). Subsequent *in vitro* work using lower concentrations of Mot1 recapitulated transcriptional stimulation, especially under conditions where excess non-promoter DNA was present (31). The authors (31) concluded that Mot1 acts by promoting dissociation of TBP from non-TATA DNA sequences and thereby raising the effective TBP concentration.

The plasmid templates used in our experiments have ~3000 base pairs of sequence, of which about 50 base pairs serve as promoter. Many suboptimal TATA boxes exist in the extraneous DNA, and we infer that TFIIA stabilizes TBP on these non-promoter sites, thus reducing the effective concentration of TBP. Our results show that BRCA1 counters TFIIA repression, and our results are consistent with this rescue occurring during preinitiation or initiation. To test whether BRCA1 could stimulate basal transcription in the absence of TFIIA, but under conditions that were unfavorable for initiation, we limited the general transcription factors involved in nucleation of the preinitiation complex, TBP and TFIIB. By limiting TBP 10-fold (from 16 to 1.6 nM), a modest stimulation of transcription by BRCA1 was revealed (Fig. 4*B*, *lanes 1* and *2*, compare with Fig. 4*A*, *lanes 1* and *2*). Under conditions where both TBP and TFIIB are limiting, the effect of BRCA1 was further enhanced (Fig. 4*B*, *lanes 3* and *4*). This result demonstrates that the stimulatory activity of BRCA1

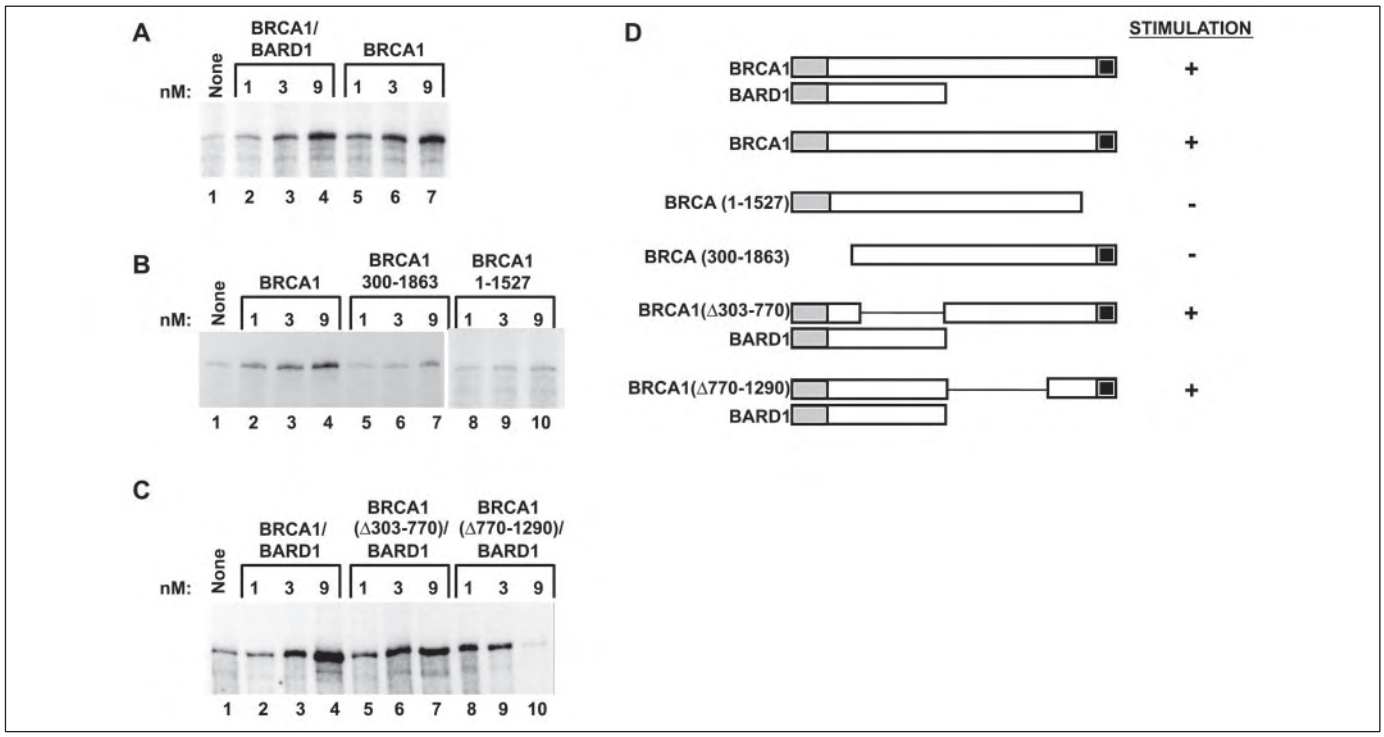

FIGURE 3. **BRCA1 amino and carboxyl termini are required for transcriptional stimulation.** *A*, transcriptional stimulation by BRCA1/BARD1 was compared with BRCA1 alone in reactions containing TFIID, TFIIA, TFIIB, RNAPII, TFIIF, TFIIE, TFIIH, and the p53 G5E4 template. The BRCA1 preparations were balanced by BRCA1 content and titrated into the reactions at 1 nM (*lanes 2* and *5*), 3 nM (*lanes 3* and *6*), and 9 nM (*lanes 4* and *7*). *B*, transcriptional stimulation by BRCA1 and the truncations BRCA1-(300–1863) and BRCA1-(1–1527) was tested as in *A*. *C*, transcriptional stimulation by BRCA1/BARD1 and internal deletions BRCA1-(Δ303–770)/BARD1 and BRCA1-(Δ770–1290)/BARD1 was tested as in *A*. *D*, summary of transcriptional stimulation by BRCA1 variants. Amino-terminal domain (*gray*) denotes RING finger domains in BRCA1 and BARD1, and the carboxyl-terminal domain (*black*) corresponds to BRCT repeats in BRCA1.

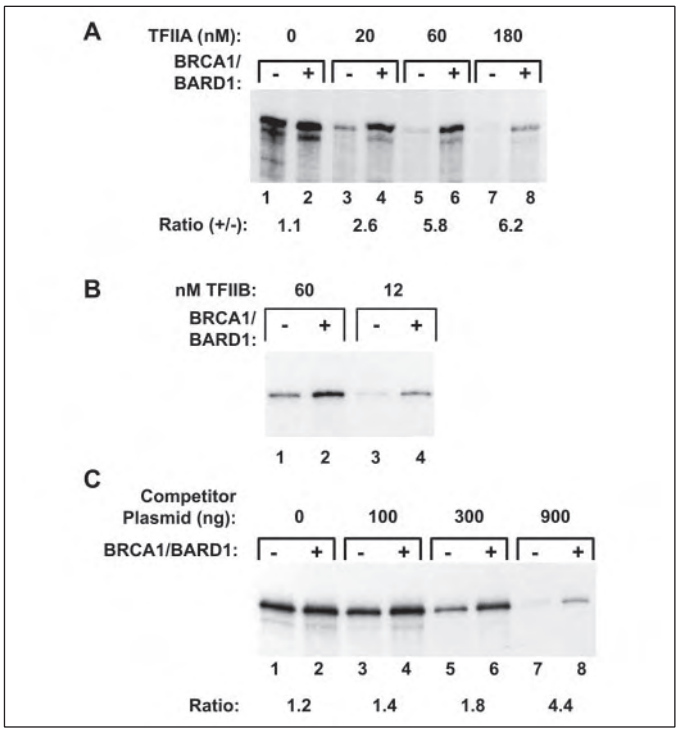

FIGURE 4. **BRCA1 promotes productive preinitiation complex formation.** *A*, TFIIA was titrated into transcription reactions containing TBP, TFIIB, RNAPII, TFIIF, TFIIE, TFIIH, and p53 G5E4 template, with BRCA1/BARD1 (10 nM) included in the *even-numbered lanes*. TFIIA concentrations were 0 nM (*lanes 1* and *2*), 20 nM (*lanes 3* and *4*), 60 nM (*lanes 5* and *6*), and 180 nM (*lanes 7* and *8*). For each concentration of TFIIA, the ratio of RNA products in transcription reactions containing BRCA1/BARD1 to without BRCA1/BARD1 was determined by PhosphorImager analysis. *B*, transcription reactions were assembled without TFIIA, containing TBP (1.6 nM), RNAPII, TFIIF, TFIIE, TFIIH, and the p53 G5E4 template. TFIIB was added at 60 nM (*lanes 1* and *2*) or 12 nM (*lanes 3* and *4*), with BRCA1/BARD1 added in the *even-numbered lanes*. *C*, competitor plasmid DNA lacking eukaryotic promoter sequences was titrated into transcription reactions utilizing p53 G5E4 template and containing TBP, TFIIB, RNAPII, TFIIF, TFIIE, and TFIIH. BRCA1/BARD1 was added to the *even-numbered lanes*. Lanes contained 0 ng of competitor plasmid (*lanes 1* and *2*), 100 ng (*lanes 3* and *4*), 300 ng (*lanes 5* and *6*), and 900 ng (*lanes 7* and *8*). The fold stimulation of transcription by BRCA1/BARD1 at each amount of plasmid addition was determined using a PhosphorImager.

is not limited to reversal of TFIIA basal repression but applies more generally to situations under which preinitiation complex assembly is a limiting step.

The challenges to proper initiation in our transcription assay likely underestimate the difficulties *in vivo*, where correct promoters must be discriminated from total genomic DNA. To test whether the presence of excess plasmid DNA could inhibit transcription, we titrated a competitor plasmid lacking promoter sequences into transcription reactions that were conducted in the presence or absence of BRCA1/BARD1 (Fig. 4*C*). With addition of 300 ng or more of competitor DNA, transcription levels were reduced, confirming that excess DNA can inhibit transcription (compare *lanes 1* and *2* with *lanes 5–8*). The most likely explanation for this effect was that the competitor DNA titrated initiation factors away from the bona fide TATA box. Although transcription levels were lower overall, we observed an increasing degree of transcriptional stimulation by BRCA1/BARD1 with increasing competitor plasmid. Without competitor DNA, the addition of BRCA1/BARD1 stimulated transcription only 1.2-fold (*lanes 1* and *2*). At the highest level of competitor plasmid tested (900 ng), RNA synthesis was stimulated by BRCA1/BARD1 over 4-fold (*lanes 7* and *8*). Therefore, the presence of excess competitor DNA inhibits transcription but increases the potential for stimulation by BRCA1.

We find that limiting the initiation factors TFIIB and TBP, either directly or by addition of excess competitor DNA, increases the stimulatory effect of BRCA1. This outcome could be explained by BRCA1 stabilization of productive initiation complexes or conversely by destabilization of non-productive complexes. Based on the known interaction between BRCA1 and RNAPII, the former possibility is, in our opinion, more likely. Taken together, our data support a model where BRCA1 stabilizes productive transcription initiation complexes, and this may be one mechanism by which it coactivates the transcription of gene targets. Stimulation by BRCA1 was observed in our assays with purified components and a range of promoters at concentrations as low as 1 nM BRCA1. However, in the cell, where BRCA1 concentration is likely even lower, it could be recruited to specific promoters by enhancer-binding factors. Once bound to a specific promoter, BRCA1 could stimulate assembly of the preinitiation complex through its interactions with RNAPII and perhaps other general transcription factors.

Acknowledgments—We thank Karen Griffin for protein purification, Lea Starita and Amanda Simons for BRCA1 truncation constructs, and Steve Buratowski and Karl Munger for helpful discussion.

REFERENCES

1. Chapman, M. S., and Verma, I. M. (1996) *Nature* **382,** 678–679
2. Monteiro, A. N., August, A., and Hanafusa, H. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 13595–13599
3. Scully, R., Anderson, S. F., Chao, D. M., Wei, W., Ye, L., Young, R. A., Livingston, D. M., and Parvin, J. D. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 5605–5610
4. Neish, A. S., Anderson, S. F., Schlegel, B. P., Wei, W., and Parvin, J. D. (1998) *Nucleic Acids Res.* **26,** 847–853
5. Zhang, H., Somasundaram, K., Peng, Y., Tian, H., Zhang, H., Bi, D., Weber, B. L., and El-Deiry, W. S. (1998) *Oncogene* **16,** 1713–1721
6. Ouchi, T., Monteiro, A. N., August, A., Aaronson, S. A., and Hanafusa, H. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 2302–2306
7. Somasundaram, K., Zhang, H., Zeng, Y. X., Houvras, Y., Peng, Y., Zhang, H., Wu, G. S., Licht, J. D., Weber, B. L., and El-Deiry, W. S. (1997) *Nature* **389,** 187–190
8. Harkin, D. P., Bean, J. M., Miklos, D., Song, Y. H., Truong, V. B., Englert, C., Christians, F. C., Ellisen, L. W., Maheswaran, S., Oliner, J. D., and Haber, D. A. (1999) *Cell* **97,** 575–586
9. Haile, D. T., and Parvin, J. D. (1999) *J. Biol. Chem.* **274,** 2113–2117
10. Krum, S. A., Womack, J. E., and Lane, T. F. (2003) *Oncogene* **22,** 6032–6044
11. Jin, S., Zhao, H., Fan, F., Blanck, P., Fan, W., Colchagie, A. B., Fornace, A. J., Jr., and Zhan, Q. (2000) *Oncogene* **19,** 4050–4057
12. Mondal, N., Zhang, Y., Jonsson, Z., Dhar, S. K., Kannapiran, M., and Parvin, J. D. (2003) *Nucleic Acids Res.* **31,** 5016–5024
13. Schlegel, B. P., Green, V. J., Ladias, J. A., and Parvin, J. D. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 3148–3153
14. Starita, L. M., Machida, Y., Sankaran, S., Elias, J. E., Griffin, K., Schlegel, B. P., Gygi, S. P., and Parvin, J. D. (2004) *Mol. Cell Biol.* **24,** 8457–8466
15. Starita, L. M., Horwitz, A. A., Keogh, M. C., Ishioka, C., Parvin, J. D., and Chiba, N. (2005) *J. Biol. Chem.* **280,** 24498–24505
16. Mondal, N., and Parvin, J. D. (2005) *Cancer Biol. Ther.* **4,** 414–418
17. Sawadogo, M., and Roeder, R. G. (1985) *Cell* **43,** 165–175
18. Parvin, J. D., Shykind, B. M., Meyers, R. E., Kim, J., and Sharp, P. A. (1994) *J. Biol. Chem.* **269,** 18414–18421
19. Parvin, J. D., and Sharp, P. A. (1993) *Cell* **73,** 533–540
20. Kaiser, K., Stelzer, G., and Meisterernst, M. (1995) *EMBO J.* **14,** 3520–3527
21. Chiba, N., and Parvin, J. D. (2002) *Cancer Res.* **62,** 4222–4228
22. Hashizume, R., Fukuda, M., Maeda, I., Nishikawa, H., Oyake, D., Yabuki, Y., Ogata, H., and Ohta, T. (2001) *J. Biol. Chem.* **276,** 14537–14540
23. Ma, D., Watanabe, H., Mermelstein, F., Admon, A., Oguri, K., Sun, X., Wada, T., Imai, T., Shiroya, T., and Reinberg, D. (1993) *Genes Dev.* **7,** 2246–2257
24. Ma, D., Olave, I., Merino, A., and Reinberg, D. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 6583–6588
25. Aso, T., Serizawa, H., Conaway, R. C., and Conaway, J. W. (1994) *EMBO J.* **13,** 435–445
26. Auble, D. T., and Hahn, S. (1993) *Genes Dev.* **7,** 844–856
27. Auble, D. T., Hansen, K. E., Mueller, C. G., Lane, W. S., Thorner, J., and Hahn, S. (1994) *Genes Dev.* **8,** 1920–1934
28. Collart, M. A. (1996) *Mol. Cell. Biol.* **16,** 6668–6676
29. Madison, J. M., and Winston, F. (1997) *Mol. Cell. Biol.* **17,** 287–295
30. Prelich, G. (1997) *Mol. Cell. Biol.* **17,** 2057–2065
31. Muldrow, T. A., Campbell, A. M., Weil, P. A., and Auble, D. T. (1999) *Mol. Cell. Biol.* **19,** 2835–2845

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 280, No. 26, Issue of July 1, pp. 24498–24505, 2005
Printed in U.S.A.

BRCA1/BARD1 Ubiquitinate Phosphorylated RNA Polymerase II*

Received for publication, December 14, 2004, and in revised form, May 4, 2005
Published, JBC Papers in Press, May 10, 2005, DOI 10.1074/jbc.M414020200

Lea M. Starita‡§, Andrew A. Horwitz‡§, Michael-Christopher Keogh¶, Chikashi Ishioka‖, Jeffrey D. Parvin‡‡, and Natsuko Chiba‖§§**

*From the ‡Program in Biology and Biomedical Sciences and the ¶Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115, the ‖Department of Clinical Oncology, Institute of Development, Aging, and Cancer and Tohoku University Hospital, Tohoku University, Sendai 980–8575, Japan, and the **Department of Pathology, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts 02115*

The breast- and ovarian-specific tumor suppressor BRCA1, when associated with BARD1, is an ubiquitin ligase. We have shown here that this heterodimer ubiquitinates a hyperphosphorylated form of Rpb1, the largest subunit of RNA polymerase II. Two major phosphorylation sites have been identified in the Rpb1 carboxyl terminal domain, serine 2 (Ser-2) or serine 5 (Ser-5) of the YSPTSPS heptapeptide repeat. Only the Ser-5 hyperphosphorylated form is ubiquitinated by BRCA1/BARD1. Overexpression of BRCA1 in cells stimulated the DNA damage-induced ubiquitination of Rpb1. Similar to the *in vitro* reaction, the stimulation of Rpb1 ubiquitination by BRCA1 in cells occurred only on those molecules hyperphosphorylated on Ser-5 of the heptapeptide repeat. *In vitro*, the carboxyl terminus of BRCA1 (amino acids 501–1863) was dispensable for the ubiquitination of hyperphosphorylated Rpb1. In cells, however, efficient Rpb1 ubiquitination required the carboxyl terminus of BRCA1, suggesting that interactions mediated by this region were essential in the complex milieu of the nucleus. These results link the BRCA1-dependent ubiquitination of the polymerase with DNA damage.

BRCA1, the breast- and ovarian-specific tumor suppressor protein, has been found to regulate a number of processes central to the normal function of the cell, including transcription, chromatin dynamics, homologous recombination, and other forms of DNA damage repair (1, 2). Because BRCA1 has been found associated with a wide range of proteins involved in these processes, it may function as a scaffold, organizing effector proteins in a context-dependent manner. However, when BRCA1 is associated with the BARD1 protein, it is also an enzyme, an E3 ubiquitin ligase (3, 4). The realization that BRCA1 is an enzyme establishes the necessity of identifying its substrates in order to understand how the ubiquitination activity impacts these processes in the cell.

BRCA1 and BARD1 are associated with the messenger RNA-synthesizing polymerase in a complex known as the RNA polymerase II holoenzyme (holo-pol)[1] (5–7). One function for BRCA1 in this holo-pol complex appears to be as a coactivator of transcription, because it has been shown that BRCA1 stimulates the activation signal of p53, NF-κB, and others (8–13). Previously, we modeled that the BRCA1 and BARD1 in the holo-pol complex may ubiquitinate the transcribing RNA polymerase II (RNAPII) when it encounters DNA damage, and we also suggested that this ubiquitination event would stimulate the repair process (14, 15).

Rpb1 is the largest subunit of RNAPII, and its carboxyl-terminal domain (CTD) is highly conserved, consisting of multiple repeats (27 in budding yeast, 52 in humans) of the heptapeptide YSPTSPS. Serines 2 (Ser-2) and 5 (Ser-5) of multiple repeats are phosphorylated co-transcriptionally, Ser5*p predominating at the promoter and Ser2*p in the coding sequence (16, 17). In response to DNA damage Rpb1 is also ubiquitinated, an event associated with changes in concentration of both the hypophosphorylated and the hyperphosphorylated Rpb1 (18). In budding yeast, the Rsp5 E3 ligase ubiquitinates Rpb1 independent of its phosphorylation state (19, 20). In higher eukaryotes the ubiquitin ligase(s) that mediate this modification of RNAPII are unknown, and it is possible that multiple factors mediate the reaction. Because BRCA1 and BARD1 are associated with RNAPII in the holo-pol complex (6), BRCA1 is a reasonable candidate for the RNAPII ubiquitin ligase. In addition, after DNA damage BRCA1 and BARD1 also associate with the polyadenylation cleavage factor CstF (21), known to interact with RNAPII via Rpb1 hyperphosphorylated on Ser-2 (Ser2*p) of the YSPTSPS heptapeptide repeats (22, 23). These results led us to speculate that a substrate for BRCA1-dependent ubiquitination could be the Ser2*p form of Rpb1.

In these experiments we tested whether BRCA1 in association with BARD1 could ubiquitinate RNAPII. We found that hyperphosphorylated RNAPII serves as a substrate for the BRCA1-dependent ubiquitination activity, and we found that overexpression of BRCA1 in cells stimulates the DNA damage-induced ubiquitination of hyperphosphorylated RNAPII. Strikingly, the ubiquitination reaction, when tested both *in vitro* and *in vivo*, was enhanced not by Ser2*p of the heptapeptide

* This work was supported in part by predoctoral fellowships from the Department of Defense Breast Cancer Research Program (to L. M. S. and A. A. H.), National Institutes of Health Grants CA90281 and GM53504 (to J. D. P.), grants-in-aid from the Ministry of Education, Science, Sports, and Culture (to N. C. and C. I.), Gonryo Medical Foundation (to N. C. and C. I.), Daiwa Securities Health Foundation (to N. C.), and Mitsui Life Social Foundation (to N. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Both authors contributed equally to this work.

‡‡ To whom correspondence may be addressed. E-mail: jparvin@rics.bwh.harvard.edu.

§§ To whom correspondence may be addressed. E-mail: nchiba@idac.tohoku.ac.jp.

[1] The abbreviations used are: holo-pol, RNA polymerase II holoenzyme; BRCA1, breast cancer gene 1; BARD1, BRCA1-associated RING domain protein 1; CTD, Rpb1 carboxyl-terminal domain; GST, glutathione *S*-transferase; RNAPII, RNA polymerase II; Rpb1, RNA polymerase II subunit 1; Ser2*p, phosphorylated serine 2 of YSPTSPS; Ser5*p, phosphorylated serine 5 of YSPTSPS; HEK, human embryonic kidney; E1, ubiquitin-activating enzyme; E2, ubiquitin carrier protein; E3, ubiquitin-protein isopeptide ligase; HA, hemagglutinin.

repeat but rather by Ser5*p. These results thus identify a substrate for ubiquitination by BRCA1/BARD1 that is correlated with the cellular response to DNA damage.

MATERIALS AND METHODS

Protein Purification—The expression and purification of BRCA1 and BARD1 from baculovirus-infected insect cells has been described, along with a description of the purification of the ubiquitination factors E1 and UbcH5c E2 (24). The core RNAPII was purified from calf thymus using an established protocol (25). The budding yeast Rpb1 CTD was expressed as a hexahistidine and GST fusion (26) and purified by nickel-nitrilotriacetic acid chromatography using standard techniques. Ubiquitin was obtained from a commercial vendor (Sigma).

The yeast Kin28, Ctk1, and Srb10 kinases were each expressed in *Saccharomyces cerevisiae* as HA-tagged fusion proteins. Active kinases were purified by immunoprecipitation using the 12CA5 monoclonal antibody specific for the HA tag (27, 28).

Human TFIIH was purified from HEK-293 cells as described (29). In brief, ~10^{12} cells were collected over a period of several months, and a whole cell extract was prepared for each. The whole cell extracts were bound to a Biorex70 matrix at 0.15 M KOAc in buffer A (20 mM Hepes, pH 7.9, 1 mM EDTA, 5% glycerol, 3 mM dithiothreitol), washed at 0.3 M KOAc, 0.6 M KOAc, and the peak was collected at 1.5 M KOAc. At each column step, TFIIH-containing fractions were identified by Western blotting using antibodies specific to the 89-kDa ERCC-3 subunit of TFIIH. The 1.5 M KOAc peak fraction was dialyzed to 0.1 M KCl in buffer A, bound to a DEAE fast flow matrix, and the protein peak at 0.3 M KCl was collected and dialyzed to 0.1 M KCl. The protein was bound to a 2-ml BioScale-Q column (Bio-Rad Laboratories), and protein was eluted in a gradient from 0.1 to 1.0 M KCl. TFIIH-containing fractions were subjected to gel filtration using a Superdex-200 (HR16/60; Amersham Biosciences) column in 0.3 M KCl in buffer A. The TFIIH migrated at a volume consistent with a 700-kDa complex, and samples were dialyzed in 0.1 M KCl in buffer A.

In Vitro Ubiquitination Assay—Purified RNAPII (10 ng) or 300 ng of GST·CTD/reaction were phosphorylated using purified human TFIIH or 12CA5 resin-bound HA-Ctk1, HA-Srb10, or HA-Kin28 kinase complexes using the following reaction conditions: 10 mM HEPES (pH 7.9), 0.5 mM EDTA, 5% glycerol, 60 mM KCl, 5 mM $MgCl_2$, 5 mM NaF, 10 μCi of [γ-^{32}P]ATP. ^{32}P-labeled RNAPII was then added to ubiquitination reactions that contained 100 ng of FLAG-BRCA1/BARD1 (25 nM) or truncations of BRCA1 co-purified with BARD1 (24), 100 ng of His_6-E1 ubiquitin ligase (40 nM), 1.5 μg of His_6-UbcH5c (4 μM), and 2 μg of ubiquitin (12 μM) in the following reaction conditions: 10 mM HEPES, pH 7.9, 5% glycerol, 60 mM KCl, 5 mM $MgCl_2$, 5 mM NaF, 2 mM ATP. All reactions were incubated at 37 °C for 30 min. The reactions were stopped by addition of sample buffer and resolved by SDS-PAGE.

Plasmid Construction—pcDNA3-HA-BRCA1(Δ775–1292)-C61G was constructed as follows. The plasmid pcDNA3-HA-BRCA1(Δ775–1292) has been described previously (30). A fragment containing the mutation C61G was amplified from an adenovirus shuttle vector that expresses full-length HA-BRCA1-C61G (31). PCR from this template used the primers 5′-ACCCCAAGCTTACCATGGCC-3′ that contains the HindIII site and 5′-TCTGTTATGTTGGCTCCTTG-3′ that is located in 3′-side of the EcoRI site of BRCA1. The PCR product was subcloned into the HindIII and EcoRI sites of pcDNA3-HA-BRCA1(Δ775–1292).

pcDNA3-HA-BRCA1(Δ775–1292) was constructed as follows. A fragment was PCR amplified from the template pcDNA3-HA-BRCA1 using the mutagenic primer 5′-GCCCTTCACCAACA*G*GCCCACAGATC-3′ and a downstream, vector-encoded primer 5′-TGACACTATAGAATAGGGCC-3′. The PCR product was used as a megaprimer with 5′-GGAAACAAAATGTTCTGCTAGCTTG-3′ to amplify a fragment encoding BRCA1 amino acids 1293–1863 containing the M1775R substitution. The second PCR product was subcloned into the NheI and EcoRV sites of pcDNA3-HA-BRCA1(Δ775–1292), thus replacing the wild-type sequence.

pcDNA3-HA-BRCA1(Δ775–1292, Δ1527–1863) was constructed as follows. The fragment containing HA-BRCA1 sequences up to residue 1526 was generated by digestion of pcDNA3-HA-BRCA1(Δ775–1292) with HindIII and SacI and then inserted into the HindIII and EcoRV sites of the vector backbone for pcDNA3-HA-BRCA1(Δ775–1292).

pCMV-Myc-ubiquitin was constructed as follows. Ubiquitin was amplified from cDNA of HeLa cells as a template using the primers 5′-GCCGAATTCGGATGCAGATCTTCGTGAAAAC-3′ and 5′-CCGCTCGAGCTAACCACCTCTCAGACGCAGG-3′ that contain 5′-EcoRI site and 3′-XhoI site. The PCR product was then subcloned into the pCMV-Myc vector (Clontech). All constructs were verified by DNA sequence.

In Vivo Ubiquitination Assay—HEK-293T cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 μg/ml penicillin and streptomycin and transfected with expression vector to express HA-BRCA1, HA-BRCA1(Δ775–1292), HA-BRCA1(Δ775–1292)-C61G, HA-BRCA1(Δ775–1292)-M1775R, and HA-BRCA1(Δ775–1292, Δ1527–1863). Two days post-transfection, cells were exposed to 20 J/m^2 of ultraviolet light and incubated with 50 μM MG132 (Sigma) in Me_2SO or Me_2SO alone for 2 h. Cell lysates were prepared in 1 ml of wash buffer (10 mM Hepes, pH 7.6, 250 mM NaCl, 0.1% Nonidet P-40, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride). For immunoprecipitation, 2.5 μl of anti-HA monoclonal antibody (HA.11; Covance), 3 μl of anti-Myc monoclonal antibody (9E10; Covance), or 7 μl of monoclonal antibody H14 and 20 μl of protein G-Sepharose beads (Amersham Biosciences) were added to each lysate. Mixtures were incubated at 4 °C overnight with rotation, the supernatant was removed, and protein beads were washed three times using 0.4 μl of wash buffer. For Western blot analysis, samples were subjected to electrophoresis in 5 or 5.5% SDS-polyacrylamide gels and immunoblotted using the monoclonal antibodies H14 or H5 (Covance), which recognize the Rpb1 CTD phosphorylated on Ser-5 or Ser-2, respectively, the anti-HA antibody HA.11, or the anti-Myc antibody 9E10.

RESULTS

BRCA1/BARD1 Ubiquitinate Hyperphosphorylated RNAPII in Vitro—BRCA1, in association with its heterodimeric partner BARD1, comprise an E3 ubiquitin ligase (3). Because BRCA1 and BARD1 associate with RNAPII (5, 7, 32), we hypothesized that RNAPII may be ubiquitinated by BRCA1/BARD1 in response to DNA damage, facilitating the repair of this damage in actively transcribed genes (14, 15).

To test this hypothesis, we utilized purified RNAPII core enzyme that had been phosphorylated *in vitro* by TFIIH as a substrate in ubiquitination reactions. Purified RNAPII exists in two forms, the IIA form, in which the Rpb1 CTD has a low level of phosphorylation, and the IIO form, in which this domain is hyperphosphorylated and has significantly shifted migration on SDS-PAGE. Phosphorylation of this RNAPII preparation by TFIIH results in the labeling of both of these forms of Rpb1 (Fig. 1*A*, *lanes 1* and *2*). This labeled RNAPII was tested in ubiquitination reactions that contained purified E1, E2 UbcH5c, E3 BRCA1/BARD1, and ubiquitin. In the complete reaction, the RNAPIIO band disappeared and a slower migrating diffuse band was observed. Under these conditions, the hypophosphorylated RNAPIIA was not modified (Fig. 1*A*, *lane 3*). These results suggest that the hyperphosphorylated RNAPII is a substrate for the BRCA1/BARD1 ubiquitin ligase.

The appearance of the slowly migrating RNAPIIO band was dependent upon the inclusion of each ubiquitination factor. Single omission of the substrate, E1, E2, E3, or ubiquitin failed to produce the slowly migrating RNAPIIO band (Fig. 1*B*). The appearance of the slowly migrating RNAPIIO band was thus consistent with modification by ubiquitination because only when all ubiquitination factors were included in reactions did this species appear (*lane 1*).

We tested whether the full 12-subunit RNAPII complex was required for ubiquitination by BRCA1/BARD1 or whether the phosphorylated CTD would suffice. The experiment of Fig. 1*B* was repeated using only the Rpb1 CTD fused to GST. This substrate was phosphorylated by purified TFIIH and [γ-^{32}P]ATP. When labeled GST·CTD was incubated with the complete reaction containing E1, E2 UbcH5c, ubiquitin and BRCA1/BARD1, the GST·CTD protein had markedly slowed migration. In this portion of the gel (>85 kDa), the resolution was imperfect, and we interpret the diffuse band with slowed migration to be consistent with the multiple additions of 8-kDa ubiquitin moieties (Fig. 1*C*, *lane 1*). The CTD of this substrate protein had no lysines to be modified by ubiquitination. We suggest that the CTD recruits the BRCA1/BARD1 E3 ligase for the ubiquitination of a separate domain of the polypeptide. These results indicate that both the 12-subunit RNAPII com-

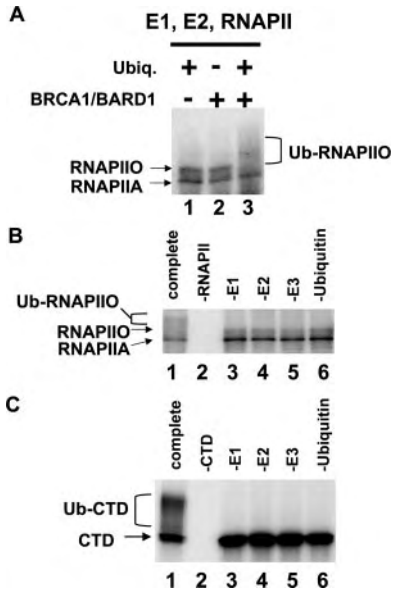

FIG. 1. **BRCA1/BARD1 ubiquitinate the Rpb1 subunit of RNAPII.** *A*, purified RNAPII was phosphorylated on the Rpb1 subunit with TFIIH and [γ-^{32}P]ATP and tested for subsequent ubiquitination using purified E1, E2 UbcH5c, E3 BRCA1/BARD1, and ubiquitin (*lane 3*). BRCA1/BARD1 and ubiquitin were included in reactions as indicated. Radiolabeled products were resolved by SDS-PAGE and identified by autoradiography. The hyperphosphorylated RNAPIIO and hypophosphorylated RNAPIIA bands are indicated. *B*, ubiquitination of RNAPII by BRCA1/BARD1 requires all of the ubiquitination factors. The complete reaction, as in *panel A*, was analyzed in *lane 1*. In *lanes 2–6* four components were included in reactions, and a different single component was omitted in each reaction. Reactions lacked RNAPII (*lane 2*), E1 (*lane 3*), E2 UbcH5c (*lane 4*), E3 BRCA1/BARD1 (*lane 5*), and ubiquitin (*lane 6*). *C*, reactions as in *panel B* were repeated except that TFIIH-phosphorylated GST·CTD was used in place of RNAPII.

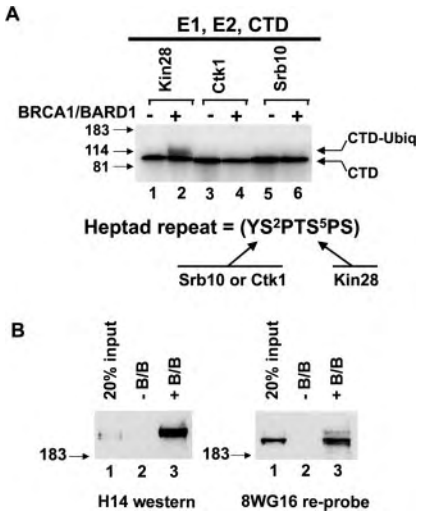

FIG. 2. **Ubiquitination of the Rpb1 CTD by BRCA1/BARD1 is stimulated by phosphorylation of the Ser-5 residue of the heptapeptide repeat.** *A*, purified GST·CTD was radiolabeled by phosphorylation with the indicated kinases. The labeling reactions create the Ser-2- or Ser-5-specific phosphopeptides, and reactions were balanced for the amount of GST·CTD and for the level of phosphorylation. After labeling, the ubiquitination reactions included E1, E2 UbcH5c, and ubiquitin. BRCA1 and BARD1 were added in reactions used in *even lanes*. *B*, purified RNAPII was subjected to affinity purification on anti-FLAG antibody containing M2-agarose beads (*lane 2*) or the same beads bound to full-length FLAG-tagged BRCA1/BARD1 protein (*B*/*B*; *lane 3*). Following binding, the matrix was washed thoroughly in buffer containing 0.3 M NaCl. Samples were analyzed by SDS-PAGE and immunoblotted with Ser5*p-specific H14 antibody (*left panel*) followed by reprobing with the RNAPIIA-specific 8WG16 antibody (*right panel*).

plex and the GST·CTD were substrates for the BRCA1/BARD1 E3 ubiquitin ligase.

The CTD used in these experiments was from the budding yeast *S. cerevisiae*, and contained 26 copies of the YSPTSPS heptapeptide. The CTD is co-transcriptionally phosphorylated *in vivo* on both Ser-2 and Ser-5. RNAPII containing unphosphorylated Rpb1 is preferentially recruited to preinitiation complexes but is phosphorylated during the transition from initiation to elongation. A Ser5*p form of the Rpb1 CTD predominates at the promoter, with Ser2*p CTD more prevalent in the coding sequence. TFIIH kinase activity is directed primarily at Ser-5 (23), with human Cdk7 and its homolog Kin28 in *S. cerevisiae* acting as the kinase in each case. The *S. cerevisiae* kinases Ctk1 and Srb10 have highest phosphorylation activity directed at Ser-2 (28). When the CTD is expressed and purified from bacteria, it is unphosphorylated, whereas RNAPII purified from eukaryotic cells is phosphorylated to different degrees on both serine positions. To test which phosphorylation event is required for ubiquitination, it was necessary to use the CTD purified from bacteria. Incubation of the CTD with each specific kinase results in differently phosphorylated products: predominantly Ser5*p when Kin28 is the kinase or Ser2*p when Ctk1 or Srb10 is used (28). We tested whether the ubiquitination activity of BRCA1/BARD1 was directed specifically at the Rpb1 CTD containing either Ser5*p or Ser2*p. In Fig. 2*A*, the GST·CTD was labeled by phosphorylation with Kin28, Ctk1, or Srb10 prior to incubation in the ubiquitination reaction. Ser5*p GST·CTD was multiply ubiquitinated in the presence of BRCA1/BARD1 (Fig. 2*A*, *lane 2*), but Ser2*p GST·CTD ubiquitination could not be detected (*lanes 4* and *6*). This result suggested that the ubiquitination of the CTD by BRCA1/BARD1 was specific for substrates containing Ser5*p.

The specificity of the BRCA1/BARD1 E3 ligase in this reaction was striking. If the heterodimer was simply binding to and ubiquitinating a long polypeptide with multiple negative charges, as in the hyperphosphorylated CTD, then we would expect little or no preference for either the Ser2*p or Ser5*p forms. Instead, the ubiquitination by BRCA1/BARD1 was specific for the Ser5*p CTD. In binding experiments using the purified BRCA1/BARD1 and purified RNAPII, we found that the BRCA1 bound to RNAPII independent of phosphorylation (Fig. 2*B*, *right panel*). This result was not surprising because it is known that BRCA1 binds to Rpb2 and Rpb12 of RNAPII (32). However, when comparing the effectiveness of the purification of RNAPII on a BRCA1/BARD1 affinity matrix, the recovery of the Ser5*p-RNAPII was more complete than was observed for the hypophosphorylated form (Fig. 2*B*, *left panel*). Thus, binding alone did not specify the ubiquitination substrate, but Ser5-specific phosphorylation enhanced both the level of binding and of ubiquitination by BRCA1/BARD1. Note that the Ser5*p form of the CTD is observed at the promoter, whereas the Ser2*p is associated with transcription elongation (17). Thus, the Ser5*p-specific modification of RNAPIIO by BRCA1/BARD1 is not consistent with targeting the elongating polymerase for ubiquitination.

BRCA1 Truncated from the Carboxyl Terminus Ubiquitinated Phosphorylated RNAPII in Vitro—The carboxyl terminus of BRCA1 (amino acids 1650–1863) associates with RNAPII via interactions with Rpb2, Rpb12, and phospho-Rpb1 subunits (7, 32). To determine whether the carboxyl terminus is required to mediate ubiquitination of RNAPII *in vitro*, we purified carboxyl-terminal truncations of BRCA1 in heterodimeric complex with full-length BARD1 (24). In addition to full-length FLAG-tagged BRCA1 (1–1863), FLAG-tagged BRCA1(1–1852), BRCA1(1–1527), BRCA1(1–1000), and BRCA1 (1–500) were coexpressed with untagged BARD1 and purified. A ΔN-BRCA1 construct (301–1863) lacking the amino-

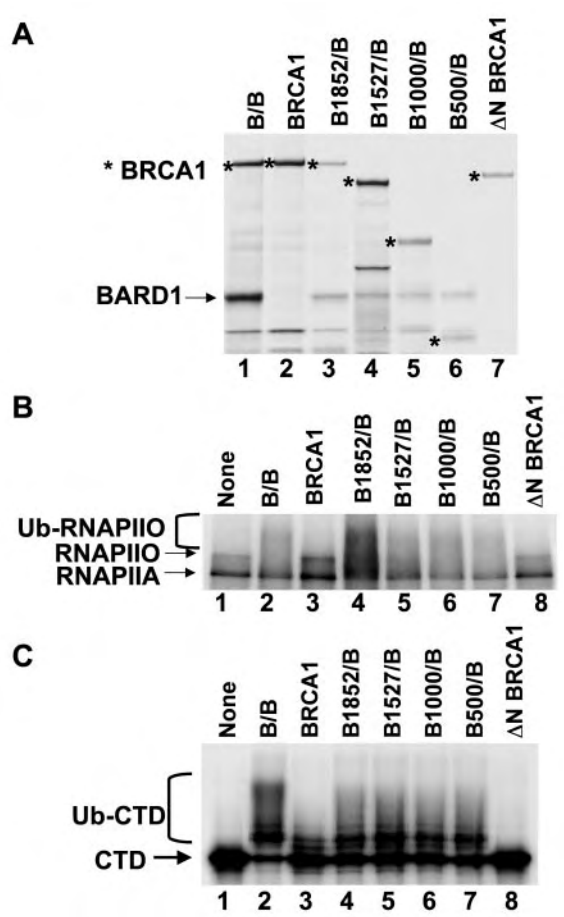

FIG. 3. **BRCA1 amino acid residues 501–1863 are dispensable for ubiquitinating phosphorylated RNAPII *in vitro*.** *A*, silver stain of a protein gel of the BRCA1/BARD1 preparations used in *panels B* and *C*. BARD1 is indicated at the side and migrated at a position consistent with a mass of 97 kDa. The BRCA1 polypeptides are marked with an *asterisk*. In each case that included BARD1, BRCA1 and BARD1 were co-expressed in insect cells and purified via an epitope tag on BRCA1. *B*, purified RNAPII, as in Fig. 1*A*, was tested as a substrate for ubiquitination using the following BRCA1 preparations: none (*lane 1*), full-length BRCA1 plus BARD1 (*lane 2*), full-length BRCA1 alone (*lane 3*), BRCA1(1–1852) plus BARD1 (*lane 4*), BRCA1(1–1527) plus BARD1 (*lane 5*), BRCA1(1–1000) plus BARD1 (*lane 6*), BRCA1 (1–500) plus BARD1 (*lane 7*), and BRCA1(301–1863) (*lane 8*). *C*, reactions as in *panel B* were repeated replacing the RNAPII complex with GST·CTD that had been labeled using TFIIH.

terminal RING domain was also purified, as was a full-length BRCA1 lacking BARD1. These constructs were balanced for BRCA1 content (Fig. 3*A*) and tested for activity in ubiquitination assays as before.

In assays using RNAPII as the substrate, ubiquitination was specific for the hyperphosphorylated Rpb1. Similar specificity was observed for all constructs tested with the exception of BRCA1 alone and the ΔN construct, which had no detectable activity. Thus, BARD1 and the BRCA1 RING domain were each required for ubiquitination of RNAPII (Fig. 3*B*). The absence of activity seen with BRCA1 lacking BARD1 is consistent with previously published results. BARD1 is required for a high level of ubiquitination activity of BRCA1, and the isolated RING domains of each protein have been shown to have low levels of ubiquitination activity *in vitro* (3, 33, 34). However, the ubiquitination activity of BRCA1 is significantly potentiated by its interaction with BARD1 (3, 4), and structural studies of the amino terminus of BRCA1 and BARD1 reveal extensive interaction between these domains (35). The ΔN construct lacks a RING domain and was thus expected to lack ubiquitination activity.

All of the active truncations of BRCA1 specifically ubiquitinated the hyperphosphorylated form of RNAPII, whereas the hypophosphorylated form was relatively unmodified (Fig. 3*B*). We had previously hypothesized that the carboxyl terminus of BRCA1 mediates the specificity of its association with RNAPII because this domain of BRCA1 activates transcription (36–38) and because it binds to two RNAPII subunits (32). Efficient ubiquitination of RNAPII, however, was observed even when the ubiquitin ligase was a BRCA1 truncation that lacked the carboxyl terminus, suggesting that the function of the BRCA1 carboxyl-terminal transcription activation domain is unrelated to its ubiquitination of phosphorylated RNAPII by BRCA1.

The RNAPII ubiquitination assay yields a qualitative result, indicating that hyperphosphorylated Rpb1 is a substrate for the ubiquitination activity of BRCA1/BARD1. We repeated the experiment using TFIIH-phosphorylated CTD (Ser5*p) as a substrate, and we found that there were no differences in the degree of ubiquitination obtained with the BRCA1 carboxyl-terminal truncations (Fig. 3*C*). Under these more sensitive conditions, weak ubiquitination was evident when BRCA1 lacking BARD1 was included in reactions (Fig. 3*C*, *lane 3*), whereas the ΔN construct had no ubiquitination activity (Fig. 3*C*, *lane 8*). Therefore, *in vitro*, the carboxyl terminus of BRCA1 is not required for ubiquitination of hyperphosphorylated RNAPII or Ser5*p-phosphorylated CTD.

BRCA1 Ubiquitinated Phosphorylated RNAPII in Vivo—We next asked whether BRCA1 could ubiquitinate hyperphosphorylated RNAPII *in vivo*. We transfected HEK-293T cells with plasmids encoding HA epitope-tagged BRCA1 and Myc epitope-tagged ubiquitin. Transfected cell lysates were immunoprecipitated using antibody specific to the Myc epitope, thus purifying ubiquitinated proteins, and then immunoblots were probed using antibodies specific to RNAPII. The immunoblot was stained with the monoclonal antibody H14, which specifically binds to RNAPII phosphorylated on Ser-5 of the heptapeptide repeat in the CTD (18). The lysate (*input*) contained a phosphorylated RNAPII large subunit that migrated at a position consistent with 240 kDa (Fig. 4*B*, *lane 1*). Background levels of ubiquitinated phospho-RNAPII were detected in cells transfected with vector alone (*lane 2*). It is established that hyperphosphorylated RNAPII becomes ubiquitinated following ultraviolet (UV) irradiation of cells (18, 39–41), and we detected the UV-dependent ubiquitination of RNAPII (Fig. 4*B*, *lane 5*). Most of the ubiquitinated species migrated on protein gels with a very small shift relative to the unmodified species (compare *lanes 5* and *1*), and this would be expected for a low number of ubiquitin moieties (about 8 kDa each) bound to a 240-kDa polypeptide. The resolution of these species was poor by SDS-PAGE, but we consistently observed stimulated recovery of the hyperphosphorylated Rpb1 band due to ubiquitination after UV irradiation. In addition, a diffuse band of ubiquitinated species was observed shifted at slower migration that we interpret to be multiply ubiquitinated RNAPIIO.

Transfection of full-length BRCA1 had minimal effect on RNAPII ubiquitination status (Fig. 4*B*, *lanes 3* and *6*). We had previously observed that overexpression of full-length BRCA1 dysregulated normal BRCA1 complex formation, presumably by altering the cell cycle (30). In those experiments, expression of a BRCA1 with an internal deletion, HA-BRCA1(Δ775–1292), allowed us to overexpress BRCA1 and observe all of the protein complexes seen with the endogenous protein (30). This internal deletion, here called HA-BRCA1(ΔM), strongly stimulated the ubiquitination of Ser5*p-hyperphosphorylated RNAPII independent of DNA damage (Fig. 4*B*, *lane 4*, *top panel*).

UV irradiation of the cells stimulated ubiquitination of phospho-RNAPII (Fig. 4*B*, *lanes 5* and *6*), and in UV-irradiated

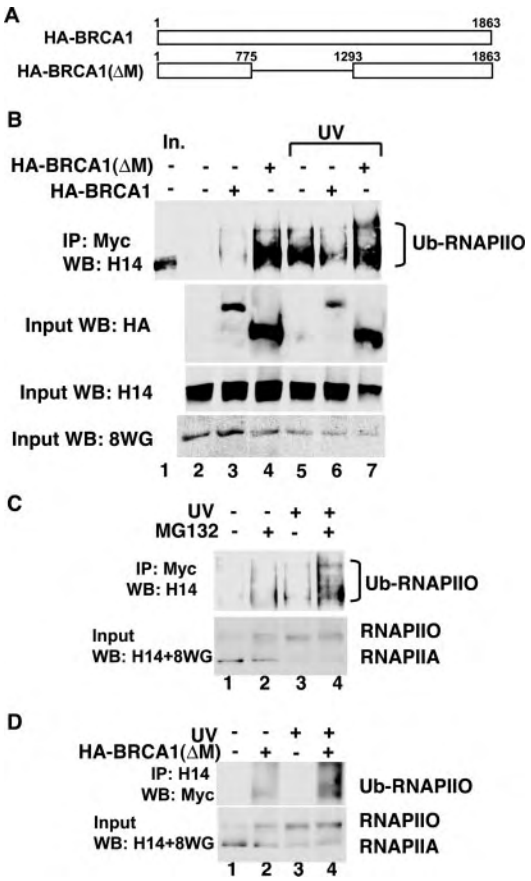

FIG. 4. **BRCA1 ubiquitinates phosphorylated RNAPII *in vivo*.** *A*, schematics are shown of the full-length BRCA1 and of the BRCA1 mutant. *B*, HEK-293T cells were transfected with vectors to express HA-BRCA1 full-length (*lanes 3*, *6*), HA-BRCA1(ΔM) (*lane 4*, *7*) and Myc ubiquitin (*lanes 1–7*). Cells were treated with 20 J/m^2 UV irradiation (*lanes 5–7*) and 50 μM MG132 (*lanes 1–7*). Lysates were immunoprecipitated by anti-Myc antibody (*lanes 2–7*). Immunoblots were stained with H14 monoclonal antibody to recognize the Ser5*p version of RNAPIIO (*top panel*). *Input* samples were immunoblotted and stained with H14, anti-HA antibody, and 8WG16 (*8WG*), the last to detect unphosphorylated RNAPII. The *input* sample (*lane 1*) was only included in the *top panel*. *C*, HEK-293T cells were transfected with vectors to express HA-BRCA1(ΔM) (*lanes 1–4*) and Myc ubiquitin (*lanes 1–4*) and treated with 20 J/m^2 UV (*lanes 3*, *4*) in the presence of 50 μM MG132 (*lanes 2*, *4*) as described under "Materials and Methods." Lysates were immunoprecipitated using an anti-Myc antibody, and immunoblots were stained for Ser5*p-RNAPIIO using antibody H14 (*top panel*). *Input* samples were stained with antibodies 8WG16 and H14 to detect hypophosphorylated RNAPIIA and Ser5*p-RNAPIIO in the samples (*bottom panel*). *D*, HEK-293T cells were transfected with vectors to express Myc ubiquitin (*lanes 1–4*) and also HA-BRCA1(ΔM) (*lanes 2* and *4*) and subjected to UV irradiation (*lanes 3* and *4*). Lysates were immunoprecipitated using the Ser5*p-specific H14 antibody and immunoblots probed with the Myc-specific antibody to detect ubiquitin (*top panel*). *Input* samples were immunoblotted using H14 and 8WG16 antibodies (*bottom panel*).

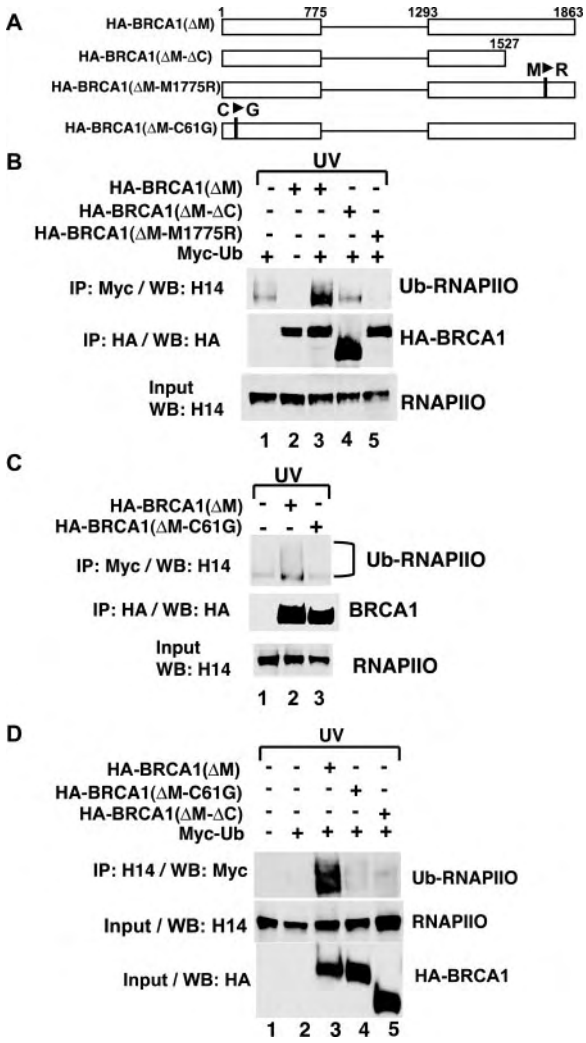

FIG. 5. **Disease-associated mutations of BRCA1 and deletion of the BRCA1 carboxyl terminus abolish BRCA1-dependent ubiquitination of phospho-RNAPII *in vivo*.** *A*, designs of BRCA1 deletion mutants and point mutations are diagrammed. *B*, BRCA1(ΔM-ΔC) and BRCA1(ΔM-M1775R) are ineffective in stimulating the ubiquitination of phosphorylated RNAPIIO. HEK-293T cells were transfected with vectors to express HA-BRCA1(ΔM) (*lanes 2*, *3*), HA-BRCA1(ΔM-ΔC) (*lane 4*), HA-BRCA1(ΔM-M1775R) (*lane 5*), and Myc ubiquitin (*lanes 1*, *3–5*). Cells were irradiated with 20 J/m^2 of UV light in the presence of 50 μM MG132, and lysates were immunoprecipitated by anti-Myc monoclonal antibody (*top panel*) or anti-HA monoclonal antibody (*middle panel*). Matching *input* samples were analyzed in the *bottom panel*. Immunoblots were stained with H14 monoclonal antibody specific for the Ser5*p-modified RNAPIIO or anti-HA monoclonal antibody as indicated. *C*, BRCA1(ΔM-C61G) was ineffective in stimulating the ubiquitination of RNAPIIO. HEK-293T cells were transfected with vectors to express HA-BRCA1(ΔM) (*lane 2*), HA-BRCA1(ΔM-C61G) (*lane 3*), and Myc ubiquitin (*lanes 1–3*). Cells were treated with 20 J/m^2 of UV irradiation in the presence of 50 μM MG132. Lysates were immunoprecipitated and immunoblotted as indicated. *D*, HEK-293T cells were transfected with plasmids expressing Myc ubiquitin (*lanes 2–5*), HA-BRCA1(ΔM) (*lane 3*), HA-BRCA1(ΔM-C61G) (*lane 4*), and HA-BRCA1(ΔM-ΔC) (*lane 5*). Cells were irradiated in the presence of MG132 as above, and lysates were immunoprecipitated using the H14 monoclonal antibody (*top panel*). *Input* lysates were analyzed in the bottom two panels, and immunoblots were probed as indicated.

HA-BRCA1(ΔM)-expressing cells a significant increase in the intensity of the slowly migrating band was observed (*lane 7*) that we interpret to be multiply ubiquitinated RNAPIIO. These results indicate that overexpression of BRCA1(ΔM) stimulated ubiquitination of Ser5*p-Rpb1 independent of, but qualitatively modified by, DNA damage. When we tested the H5 monoclonal antibody that specifically binds to Ser2*p RNAPII or the 8WG16 monoclonal antibody that specifically recognizes hypophosphorylated RNAPII on immunoblots, ubiquitinated RNAPII was not detected (data not shown). These results were consistent with the *in vitro* experiments (Fig. 2) in which Ser-5 phosphorylation of the RNAPII CTD specifically stimulated its ubiquitination by BRCA1/BARD1. These results were also consistent with the previously established ubiquitination of Ser-5-phosphorylated RNAPIIO after UV-induced DNA damage (18, 40).

The consequences of BRCA1-dependent ubiquitination are unclear. BRCA1/BARD1 have been shown to direct the linkage of ubiquitin chains via either lysine 6, lysine 48, or lysine 63 isopeptide bonds (4, 42). Appending ubiquitin chains via lysine

48 target the substrate for proteasome-mediated degradation; thus BRCA1/BARD1 ubiquitination may in some cases not lead to protein degradation. We tested whether inhibition of the proteasome, using MG132, could stabilize the ubiquitinated phospho-RNAPII. Proteasome inhibition resulted in longer chains of ubiquitin appended on the Rpb1 subunit of RNAPIIO (Fig. 4*C*, *lane 4*, *top panel*), suggesting that BRCA1-dependent ubiquitination may cause degradation of RNAPIIO. Interestingly, UV irradiation of cells resulted in a shift in the polymerase from RNAPIIA to RNAPIIO (Fig. 4*C*, *bottom panel*), a phenomenon that has been observed previously (18). Although quantitation using two different antibodies in immunoblots is imprecise, this result suggests that phosphorylation of Rpb1 to Ser5*p is a generalized response after DNA damage. Although proteasome inhibition stabilized the recovery of ubiquitinated RNAPIIO (*lanes 3* and *4*), the amount of RNAPIIO in the lysate was not markedly increased (Fig. 4*C*, *lanes 3–4*, *bottom panel*). We infer from this result that only a fraction of the total RNAPII is targeted for degradation following BRCA1-dependent ubiquitination.

Repeating the experiment, but using the H14 antibody to immunoprecipitate the RNAPIIO and the anti-Myc antibody on immunoblots to detect the ubiquitin, revealed that HA-BRCA1(ΔM) expression stimulated the appearance of ubiquitinated RNAPIIO (Fig. 4*D*, *lane 2*). As in *panel B*, expression of HA-BRCA1(ΔM) in UV-irradiated cells resulted in the recovery of higher levels of ubiquitinated RNAPIIO (Fig. 4*D*, *lane 4*). Compared with anti-Myc ubiquitin immunoprecipitation, use of the H14 antibody reproducibly yielded lower amounts of ubiquitinated RNAPIIO, even after UV irradiation. We interpret this lower level to be due to less effective immunoprecipitation reactions with the latter antibody.

We have previously shown that BRCA1 is a component of RNAPII holo-pol, and the carboxyl terminus of BRCA1 is important for this association (5, 6). In the *in vitro* assays in this study (Fig. 3), the carboxyl terminus of BRCA1 was not required for ubiquitination of the polymerase. However, in the complicated environment of a cell, the carboxyl-terminal-mutated BRCA1 might not associate with the polymerase and thus not ubiquitinate it. We examined whether the carboxyl terminus of BRCA1 affected ubiquitination of phospho-RNAPII in tissue culture cells. We found that overexpression of BRCA1 lacking its carboxyl terminus resulted in only background levels of ubiquitinated RNAPIIO (Fig. 5*B*, compare *lanes 1–4*). We thus conclude that in cells the carboxyl terminus of BRCA1 is important for the UV damage-induced ubiquitination of RNAPIIO.

We also tested whether a specific missense mutation associated with breast cancer affects the ubiquitination of RNAPIIO. The disease-associated missense mutation M1775R in the BRCT domain of the carboxyl terminus of BRCA1 ablates the double strand break repair and transcription activation function of BRCA1 (43). BRCA1 proteins containing the M1775R mutation do not bind to histone deacetylases (44), BACH1 (45), and the transcriptional co-repressor CtIP (46, 47). As shown in Fig. 5*B*, expression of BRCA1 with M1775R did not stimulate the ubiquitination of phosphorylated RNAPII (Fig. 5*B*, *lane 5*, *top panel*). Although the mutation of BRCA1 at residue M1775R decreases the stability of the protein (48), the expression level of the HA-BRCA1(ΔM-M1775R) was equal to that of HA-BRCA1(ΔM) (Fig. 5*B*, *middle panel*). Furthermore, the M1775R mutation disrupted BRCA1 binding to RNAPIIO (Fig. 6). In transfected cells, immunopurification of HA-BRCA1(ΔM) also purified Ser5*p Rpb1 (Fig. 6, *lane 2*). Deletion of the carboxyl terminus of BRCA1 or the BRCA1 protein containing a missense mutation resulted in significantly decreased binding to RNAPIIO (Fig. 6, *lanes 3* and *4*). Thus, an intact carboxyl terminus was required for BRCA1 to bind to RNAPIIO. These data suggest that ubiquitination of phosphorylated RNAPII by BRCA1 in response to DNA damage requires an intact BRCT domain.

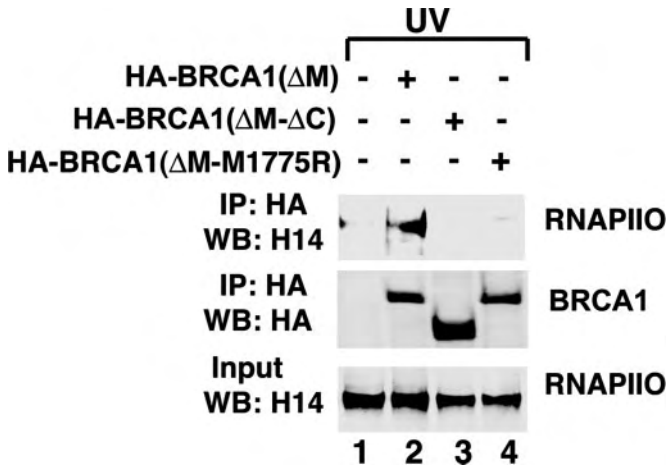

FIG. 6. **Wild-type BRCA1 binds to RNAPIIO.** HEK-293T cells were transfected with plasmids for the expression of HA-BRCA1(ΔM) (*lane 2*), HA-BRCA1(ΔM-ΔC) (*lane 3*), HA-BRCA1(ΔM-M1775R) (*lane 4*), and Myc-ubiquitin (*lanes 1–4*) and treated with 20 J/m^2 UV (*lanes 1–4*). Lysates were immunoprecipitated using anti-HA epitope antibody (*top* and *middle panels*) and probed for immunoblots as indicated. *Input* samples were analyzed in the *bottom panel*.

The active site of BRCA1 for ubiquitin ligase activity is encoded in the RING domain of the amino terminus of the protein. Missense mutation of one of the zinc-coordinating residues, C61G, results in an inactive E3 ubiquitin ligase even in the presence of wild-type BARD1 (3, 34, 35). In patients, inheritance of this missense mutation is associated with breast cancer (49, 50). Expression of HA-BRCA1(ΔM) containing the C61G missense mutation did not stimulate the ubiquitination of phosphorylated RNAPII (Fig. 5*C*, *top panel*).

The experiment in Fig. 5*C* was repeated, but the immunoprecipitating antibody was the Ser5*p-specific H14, and ubiquitinated species were detected using the Myc-specific antibody on immunoblots. As before, we observed that HA-BRCA1(ΔM) expression stimulated the recovery of ubiquitinated RNAPIIO (Fig. 5*D*, *lane 3*). Further, expression of BRCA1 variants containing the missense mutation C61G (*lane 4*) or a carboxyl-terminal truncation (*lane 5*) failed to stimulate the ubiquitination of RNAPIIO. As in Fig. 4*D*, this immunoprecipitation reaction was weaker than when the Myc antibody was used, and we only detected the ubiquitinated species when HA-BRCA1(ΔM) was expressed. Taken together, the data in Figs. 4 and 5 indicate that BRCA1 stimulates the ubiquitination of Ser5*p RNAPII after UV irradiation.

DISCUSSION

Identification of the substrates for BRCA1-dependent ubiquitination activity is important for understanding how mutation of BRCA1 is associated with loss of tumor suppression activity. The currently identified substrates include histone proteins, p53, Fanconi anemia protein D2, and centrosomal proteins including NPM1 and γ-tubulin (24, 51–54). Among these, only the modification of γ-tubulin by BRCA1/BARD1 has been shown to affect the biology of breast cells. It has been shown that failure to ubiquitinate γ-tubulin results in centrosome amplification (24). The BRCA1/BARD1 proteins are known to regulate multiple processes in the cell, including transcription, DNA repair, and centrosome dynamics (5, 55–59). Although the ubiquitination of γ-tubulin may in part ex-

plain the BRCA1-dependent regulation of centrosome dynamics, it was unclear whether the BRCA1-dependent ubiquitination activity also regulates the transcription and DNA repair function of BRCA1.

We had proposed that the BRCA1-dependent ubiquitination activity may function in DNA repair by modification of RNAPII that transcribes DNA near a lesion (14, 15). This proposed role for BRCA1 in transcription-coupled repair could be important following UV damage or double strand breaks. One prediction of this model was that BRCA1/BARD1 ubiquitination activity would be targeted to the elongating, hyperphosphorylated form of RNAPII. Actively transcribing RNAPII is phosphorylated on Ser-5 proximal to the promoter and on Ser-2 further downstream (23). Thus, the principal form of RNAPII that elongates through a gene is the Ser2*p form, which we now show is not a substrate for BRCA1/BARD1. The model that BRCA1-dependent ubiquitination directly links transcription elongation to repair is thus not supported. Instead, we found that Ser-5 phosphorylation of RNAPII is a generalized response to UV irradiation, and BRCA1-dependent ubiquitination modifies the RNAPIIO. It has been observed that transcriptionally engaged RNAPII does become phosphorylated on Ser-5 by the action of extracellular signal-regulated kinases 1 and 2 (60). The data are most consistent with a model whereby DNA damage causes phosphorylation of a subpopulation of RNAPII, followed by ubiquitination by BRCA1/BARD1 and subsequent degradation at the proteasome.

In these experiments we found that overexpression of BRCA1 in cells could stimulate the damage-induced ubiquitination of RNAPII. When we inhibited BRCA1 expression by transfection of short interfering RNA specific for BRCA1, we did not observe a decrease in ubiquitination of RNAPII.[2] We interpret these results to indicate that one or more other ubiquitin ligases can execute this function. Several other factors have been implicated in the ubiquitination of RNAPII, including Cockayne syndrome proteins CSA and CSB (60, 61). Even though other factors can also ubiquitinate RNAPII, our results overexpressing BRCA1 clearly indicate that it participates in this process.

In summary, we found in this study that BRCA1/BARD1 ubiquitinate RNAPII hyperphosphorylated via Ser-5 of the heptapeptide repeat. Rpb1 was multiply ubiquitinated. In experiments using highly purified factors *in vitro*, only the amino terminus of BRCA1, containing the catalytic RING domain, was required for ubiquitination of phospho-RNAPII. The BARD1 protein was not essential, but it was highly stimulatory. In cells, overexpression of BRCA1 could stimulate the ubiquitination of hyperphosphorylated RNAPII. In contrast to the *in vitro* reactions using purified factors, in the cell the carboxyl-terminal domain was important for the DNA damage-stimulated ubiquitination of phosphorylated RNAPII by BRCA1. These results are consistent with our observations that both the amino- and carboxyl-terminal domains of BRCA1 are required for BRCA1 association with the polymerase complex.

Acknowledgments—We thank K. Griffin for technical assistance in these experiments. We also thank Dr. S. Buratowski for helpful discussion.

REFERENCES

1. Venkitaraman, A. R. (2002) *Cell* **108,** 171–182
2. Tutt, A., and Ashworth, A. (2002) *Trends Mol. Med.* **8,** 571–576
3. Hashizume, R., Fukuda, M., Maeda, I., Nishikawa, H., Oyake, D., Yabuki, Y., Ogata, H., and Ohta, T. (2001) *J. Biol. Chem.* **276,** 14537–14540
4. Xia, Y., Pao, G. M., Chen, H. W., Verma, I. M., and Hunter, T. (2003) *J. Biol. Chem.* **278,** 5255–5263
5. Scully, R., Anderson, S. F., Chao, D. M., Wei, W., Ye, L., Young, R. A., Livingston, D. M., and Parvin, J. D. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 5605–5610
6. Chiba, N., and Parvin, J. D. (2002) *Cancer Res.* **62,** 4222–4228
7. Krum, S. A., Miranda, G. A., Lin, C., and Lane, T. F. (2003) *J. Biol. Chem.* **278,** 52012–52020
8. Houvras, Y., Benezra, M., Zhang, H., Manfredi, J. J., Weber, B. L., and Licht, J. D. (2000) *J. Biol. Chem.* **275,** 36230–36237
9. Benezra, M., Chevallier, N., Morrison, D. J., MacLachlan, T. K., El-Deiry, W. S., and Licht, J. D. (2003) *J. Biol. Chem.* **278,** 26333–26341
10. Hu, Y. F., and Li, R. (2002) *Genes Dev.* **16,** 1509–1517
11. MacLachlan, T. K., Takimoto, R., and El-Deiry, W. S. (2002) *Mol. Cell. Biol.* **22,** 4280–4292
12. Ouchi, T., Monteiro, A. N., August, A., Aaronson, S. A., and Hanafusa, H. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 2302–2306
13. Zhang, H., Somasundaram, K., Peng, Y., Tian, H., Bi, D., Weber, B. L., and El-Deiry, W. S. (1998) *Oncogene* **16,** 1713–1721
14. Starita, L. M., and Parvin, J. D. (2003) *Curr. Opin. Cell Biol.* **15,** 345–350
15. Parvin, J. D. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 5952–5954
16. Komarnitsky, P., Cho, E. J., and Buratowski, S. (2000) *Genes Dev.* **14,** 2452–2460
17. Cho, E. J., Kobor, M. S., Kim, M., Greenblatt, J., and Buratowski, S. (2001) *Genes Dev.* **15,** 3319–3329
18. Bregman, D. B., Halaban, R., van Gool, A. J., Henning, K. A., Friedberg, E. C., and Warren, S. L. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 11586–11590
19. Beaudenon, S. L., Huacani, M. R., Wang, G., McDonnell, D. P., and Huibregtse, J. M. (1999) *Mol. Cell. Biol.* **19,** 6972–6979
20. Huibregtse, J. M., Yang, J. C., and Beaudenon, S. L. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 3656–3661
21. Kleiman, F. E., and Manley, J. L. (2001) *Cell* **104,** 743–753
22. Bentley, D. (1998) *Nature* **395,** 21–22
23. Buratowski, S. (2003) *Nat. Struct. Biol.* **10,** 679–680
24. Starita, L. M., Machida, Y., Sankaran, S., Elias, J. E., Griffin, K., Schlegel, B. P., Gygi, S. P., and Parvin, J. D. (2004) *Mol. Cell. Biol.* **24,** 8457–8466
25. Thompson, N. E., Aronson, D. B., and Burgess, R. R. (1990) *J. Biol. Chem.* **265,** 7069–7077
26. Chao, D. M., Gadbois, E. L., Murray, P. J., Anderson, S. F., Sonu, M. S., Parvin, J. D., and Young, R. A. (1996) *Nature* **380,** 82–85
27. Keogh, M. C., Cho, E. J., Podolny, V., and Buratowski, S. (2002) *Mol. Cell. Biol.* **22,** 1288–1297
28. Keogh, M. C., Podolny, V., and Buratowski, S. (2003) *Mol. Cell. Biol.* **23,** 7005–7018
29. Mondal, N., and Parvin, J. D. (2001) *Nature* **413,** 435–438
30. Chiba, N., and Parvin, J. D. (2001) *J. Biol. Chem.* **276,** 38549–38554
31. You, F., Chiba, N., Ishioka, C., and Parvin, J. D. (2004) *Oncogene* **23,** 5792–5798
32. Schlegel, B. P., Green, V. J., Ladias, J. A., and Parvin, J. D. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 3148–3153
33. Lorick, K. L., Jensen, J. P., Fang, S., Ong, A. M., Hatakeyama, S., and Weissman, A. M. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 11364–11369
34. Ruffner, H., Joazeiro, C. A., Hemmati, D., Hunter, T., and Verma, I. M. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 5134–5139
35. Brzovic, P. S., Rajagopal, P., Hoyt, D. W., King, M. C., and Klevit, R. E. (2001) *Nat. Struct. Biol.* **8,** 833–837
36. Monteiro, A. N., August, A., and Hanafusa, H. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 13595–13599
37. Haile, D. T., and Parvin, J. D. (1999) *J. Biol. Chem.* **274,** 2113–2117
38. Chapman, M. S., and Verma, I. M. (1996) *Nature* **382,** 678–679
39. Mitsui, A., and Sharp, P. A. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 6054–6059
40. Lee, K. B., Wang, D., Lippard, S. J., and Sharp, P. A. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 4239–4244
41. Ratner, J. N., Balasubramanian, B., Corden, J., Warren, S. L., and Bregman, D. B. (1998) *J. Biol. Chem.* **273,** 5184–5189
42. Wu-Baer, F., Lagrazon, K., Yuan, W., and Baer, R. (2003) *J. Biol. Chem.* **278,** 34743–34746
43. Scully, R., Ganesan, S., Vlasakova, K., Chen, J., Socolovsky, M., and Livingston, D. M. (1999) *Mol. Cell* **4,** 1093–1099
44. Yarden, R. I., and Brody, L. C. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 4983–4988
45. Cantor, S. B., Bell, D. W., Ganesan, S., Kass, E. M., Drapkin, R., Grossman, S., Wahrer, D. C., Sgroi, D. C., Lane, W. S., Haber, D. A., and Livingston, D. M. (2001) *Cell* **105,** 149–160
46. Yu, X., Wu, L. C., Bowcock, A. M., Aronheim, A., and Baer, R. (1998) *J. Biol. Chem.* **273,** 25388–25392
47. Li, S., Chen, P. L., Subramanian, T., Chinnadurai, G., Tomlinson, G., Osborne, C. K., Sharp, Z. D., and Lee, W. H. (1999) *J. Biol. Chem.* **274,** 11334–11338
48. Williams, R. S., and Glover, J. N. (2003) *J. Biol. Chem.* **278,** 2630–2635
49. Friedman, L. S., Ostermeyer, E. A., Szabo, C. I., Dowd, P., Lynch, E. D., Rowell, S. E., and King, M. C. (1994) *Nat. Genet.* **8,** 399–404
50. Castilla, L. H., Couch, F. J., Erdos, M. R., Hoskins, K. F., Calzone, K., Garber, J. E., Boyd, J., Lubin, M. B., Deshano, M. L., Brody, L. C., Collins, F. S., and Weber, B. (1994) *Nat. Genet.* **8,** 387–391
51. Chen, A., Kleiman, F. E., Manley, J. L., Ouchi, T., and Pan, Z. Q. (2002) *J. Biol. Chem.* **277,** 22085–22092
52. Dong, Y., Hakimi, M. A., Chen, X., Kumaraswamy, E., Cooch, N. S., Godwin, A. K., and Shiekhattar, R. (2003) *Mol. Cell* **12,** 1087–1099
53. Vandenberg, C. J., Gergely, F., Ong, C. Y., Pace, P., Mallery, D. L., Hiom, K., and Patel, K. J. (2003) *Mol. Cell* **12,** 247–254
54. Sato, K., Hayami, R., Wu, W., Nishikawa, T., Nishikawa, H., Okuda, Y., Ogata, H., Fukuda, M., and Ohta, T. (2004) *J. Biol. Chem.* **279,** 30919–30922
55. Scully, R., Chen, J., Plug, A., Xiao, Y., Weaver, D., Feunteun, J., Ashley, T.,

[2] L. M. Starita, unpublished observations.

and Livingston, D. M. (1997) *Cell* **88,** 265–275
56. Snouwaert, J. N., Gowen, L. C., Latour, A. M., Mohn, A. R., Xiao, A., DiBiase, L., and Koller, B. H. (1999) *Oncogene* **18,** 7900–7907
57. Moynahan, M. E., Chiu, J. W., Koller, B. H., and Jasin, M. (1999) *Mol. Cell* **4,** 511–518
58. Hsu, L. C., and White, R. L. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 12983–12988
59. Xu, X., Weaver, Z., Linke, S. P., Li, C., Gotay, J., Wang, X. W., Harris, C. C., Ried, T., and Deng, C. X. (1999) *Mol. Cell* **3,** 389–395
60. Inukai, N., Yamaguchi, Y., Kuraoka, I., Yamada, T., Kamijo, S., Kato, J., Tanaka, K., and Handa, H. (2004) *J. Biol. Chem.* **279,** 8190–8195
61. Svejstrup, J. Q. (2003) *J. Cell Sci.* **116,** 447–451